Volume 16 JANUARY-FEBRUARY, 1951 Number 1

Food Research

An Official Publication of

The Institute of Food Technologists

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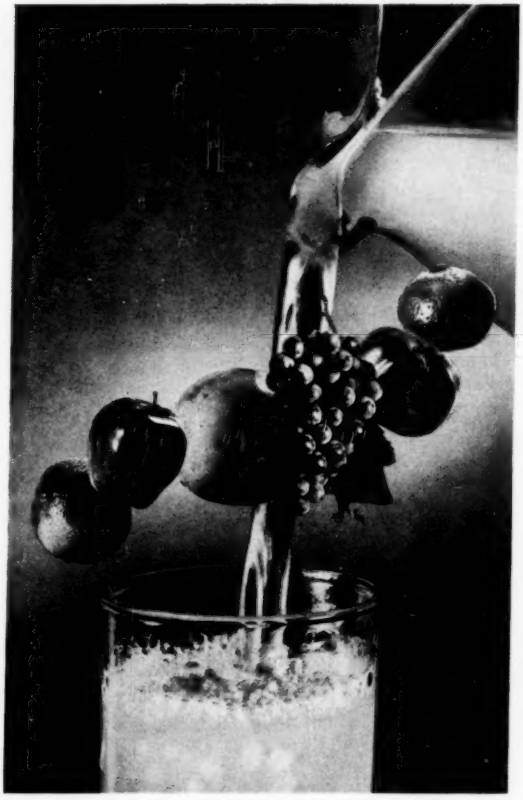

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MODE OF ACTION OF SODIUM BENZOATE [1]

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Sodium benzoate has been used as a food preservative for many years. While its use in food was the center of a long controversy well documented in the U. S. Department of Agriculture Report No. 88, and Bureau of Chemistry Circular No. 39, the reason for the effectiveness of sodium benzoate as a food preservative has never been elucidated. Certain new developments have disclosed the mode of action of certain substances to be antimetabolic. It seemed possible that the action of sodium benzoate might be explained similarly.

The concept of antimetabolites had its beginning when Elvehjem, *et al.* (1937) while working with nicotinic acid deficient animals observed that 3-acetylpyridine, though similar in structure to nicotinic acid, acted as a poison to the test animals. Later Fildes (1940) and Woods (1940) established that sulfanilamide owed its effectiveness to the manner in which it blocked bacterial use of p-aminobenzoic acid, an essential nutrilite. Work of this nature was quickly taken up by Wooley (1946, 1949), Welch (1945), Roblin (1946), and Hirsch (1942).

Wyss (1941) established the sulfonamide p-aminobenzoic acid relationship mathematically. Hartelius and Roholt (1946) later proved that there were many factors involved in this relationship beyond the sulfanilamide concentration, including age of the cells, inoculum size, and length of test period. This work was expanded to include other metabolites. Wright and Skeggs (1946) were able to reverse the inhibitory effect of sodium propionate on *E. coli* by the addition of beta-alanine. Hoffman, Schweitzer, and Dalby (1941, 1942) investigated the relationship between ring placement of hydroxy groups in aminobenzoic acids and fungistatic properties, and found the o-aminobenzoic acid (salicylic acid) to be slightly less fungistatic than benzoic acid, while the fungistatic properties were lost when the hydroxy group was placed in the meta or para position. Recent reviews of interest are those by Knight (1949) and Roblin (1949). Present theories postulate that antimetabolites function either by preventing incorporation of a metabolite into a more complex system as exemplified by the sulfonamides upon p-aminobenzoic acid and folic acid, or by being themselves incorporated into an analog of a vitamin coenzyme which then competes directly with the true coenzyme for the apoenzyme.

Since it was anticipated that an antimetabolite effect would be manifest through rendering unavailable one of the growth factors for yeast, a search of the voluminous literature on yeast growth factors was made. Several excellent reviews of this subject

[1] Presented at the Tenth Annual Meeting of the I.F.T., Chicago, Illinois, May 23, 1950.

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have been written including those by Knight (1944), Williams (1933), Burkholder, *et al.* (1944), Tanner (1925), Atkin (1949), and Koser and Saunders (1938). In addition to the reviews there has been a large volume of work done on the individual growth factors. These include the thorough studies of "Wildiers' Bios" by Miller and co-workers (1939), the study on vitamin B_6 by Eakin and Williams (1939), of inositol, thiamin, biotin, pantothenic acid, and vitamin B_6 by Williams, *et al.* (1940), and the studies on thiamin, pyridoxine, inositol, pantothenic acid, and biotin by Leonian and Lilly (1942). Rogosa (1943) studied the nicotinic acid requirements of lactose and non-lactose fermenting yeasts, and Tanner and Millhouse (1926) studied the growth promoting substances in yeast water. Many other pertinent studies have been made on yeast growth factors which have been omitted here for the sake of brevity. For further information the reviews listed above should be consulted.

Para-aminobenzoic acid, hereafter called PABA, was selected as a likely nutrilite to be competitive with sodium benzoate because of the close similarity in chemical configuration. The role of PABA in cell metabolism has not yet been fully established. Rosenberg (1945) has stated that little is known of the physiology of PABA, but that in common with the sulfonamides it is involved in some enzyme system. Ansbacher (1944) has suggested that it functions in man by aiding the growth of intestinal bacteria which in turn synthesize needed substances. He listed PABA as being not essential for the growth of animals, but capable of producing an inositol deficiency when fed in large amounts. He had previously (1941) established that PABA could cure achromotrichia in piebald rats. Scott and Robbins (1942) studied the toxicity of PABA and found that in dogs, doses of one gm. per kg. led to death from internal hemorrhages. However, PABA has been found to be an essential growth factor for *Br. abortus* and certain other organisms by Green (1940); for a number of organisms by Landy, *et al.* (1943); for *Acet. suboxydans* by Landy and Streightoff (1943) and Lampen, *et al.* (1942); for *Cl. acetobutylicum* by Lampen and Peterson (1941); Park and Wood (1942), and Rubbo and Gillespie (1940); for *Strep. haemolyticus* by Selbie (1940); and for certain English top fermenting brewers' yeasts by Rainbow (1948). The picture is further complicated by the fact that certain organisms require PABA, but are also capable of synthesizing it, as shown in studies by Lampen, *et al.* (1943), and Landy, *et al.* (1943). The requirement of certain organisms for PABA has been employed as the basis for quantitative estimation methods. Tatum and Beadle (1942) used an X-ray induced mutant of *Neurospora crassa*, and Landy and Dicken (1942) used *Acet. suboxydans*. Lewis (1942) has described a PABA assay method using *Lact. arabinosus*. Roblin (1949) described the relationship of PABA to the folic acid molecule.

While the precise mode of action of sodium benzoate has not been clearly elucidated, there have been many postulations. Wyss (1948) in a discussion of food preservatives considered the benzoates as a group and explained their action as either an effect upon the cell membrane, or competition with a coenzyme for the enzyme protein. Fitzgerald and Bernheim (1947) found that benzoic and salicylic acids greatly increased the metabolic rate of mycobacteria, but were themselves not oxidized. Therefore they felt that the acids probably catalyzed some endogenous metabolic reaction. Macht (1926) showed the unexpected bolstering effect of light upon benzoate inhibition of yeast. Roholt (1946) conducted an exhaustive study of the effect of benzoic acid and its derivatives upon *Strep. plantarum*, and *S. cerevisiae*. He found that the inhibitory effect of meta-aminobenzoic acid upon *Strep. plantarum* was reversed by PABA.

EXPERIMENTAL PROCEDURE

Medium. In a preliminary study several media were examined in combination with several strains of yeast. An original medium (Table 1) proved to be the best growth substrate when compared with the media described by Williams and Saunders (1934), Lones (1948), Wickerham (1946), and Burkholder (1944). The yeast strains are

described in Table 2. To simplify the preparation of Medium A, which was the one selected for further study, concentrates were prepared. The concentrates described below were selected because they produced no precipitation upon mixing.

Ferric chloride, copper sulfate, and potassium iodide were prepared in a mixed hundredfold concentration, all other ingredients were prepared in tenfold concentrates, except sucrose, ammonium sulfate, monopotassium acid phosphate, asparagine, and magnesium sulfate which were added as dry ingredients to each batch as prepared. All vitamins and amino acids were mixed in the same concentrate, the minerals not mentioned above were mixed in another concentrate. All concentrates were stored at −18°C. after being packaged in 250 ml. amounts in "medicine-type" bottles and tightly sealed. For use, the concentrate was thawed, warmed to room temperature, and thoroughly mixed. Freezing had no effect upon the nutritive value of the medium. Media which had been stored for three months and thawed several times were compared to a freshly prepared unfrozen batch and found to furnish equivalent growth value.

TABLE 1

Media Studied for Yeast Growth

	A	B
	gm.	*gm.*
Sucrose	20.0	20.0
Ammonium sulfate	3.0	2.0
Monopotassium phosphate	2.0	1.5
Asparagine	0.1	1.0
Calcium chloride	0.25	0.33
Magnesium sulfate	0.25	0.5
	mg.	*mg.*
Thallium chloride	1.0	1.0
Zinc sulfate	1.0	1.0
Manganese chloride	1.0	1.0
Boric acid	1.0	1.0
Ferric chloride	0.5	0.5
Copper sulfate	0.1	0.1
Potassium iodide	0.1	0.1
Inositol	5.0	5.0
Thiamin	0.5	0.5
Biotin	0.002	0.002
Calcium pantothenate	0.5	0.5
Pyridoxine	0.5	0.5
Beta alanine	0.5	0.5
Nicotinic acid	0.5	0.5
Para-aminobenzoic acid	0.5	0.5
l-Lysine	25.0	25.0
l-Arginine	50.0	50.0
l-Glutamic acid	100.0	100.0
dl-Methionine	15.0	15.0
Tryptophane	10.0	10.0
Folic acid	0.5	0.5
Riboflavin	0.2	0.2
Sodium chloride	100.0	100.0

Make up to 1 L, adjust to pH 45.

The desired amounts were pipetted into a large beaker, and the dry ingredients were dissolved therein. The pH was adjusted to 4.5 by means of 0.1 N sodium hydroxide or hydrochloric acid with the aid of a Beckman pH meter. The medium was then made up to one liter, thoroughly mixed, and distributed in five ml. amounts in previously matched colorimeter tubes supplied with the Klett-Summerson Photoelectric Colorimeter. All tubes were closed by Aloe-Willett Culture Caps and sterilized by autoclaving at

15 pounds' pressure for 15 minutes. Time and temperature of sterilization were found to be extremely critical.

Yeast Strains. The reaction of the yeast strains studied to medium A was determined (Table 2). *S. ellipsoideus*, Jerez No. 5, hereafter called Jerez No. 5, and *S. cerevisiae*, bakers' (National Yeast Company Active Dried Strain), hereafter called NYD were selected for use in the studies which follow.

TABLE 2

Comparative Growth of Yeast Strains on Selected Medium

Yeast	Strain	Source	Growth on Medium A
S. ellipsoideus	Tokay	Joslyn—U. of California	Good
S. ellipsoideus	Burgundy	Joslyn—U. of California	Good
S. ellipsoideus	Champagne	Joslyn—U. of California	Good
S. ellipsoideus	Jerez No. 5	Berkeley Yeast Laboratory	Excellent
S. cerevisiae	Dried bakers'	National Yeast Company	Excellent
S. spp.	?	Spoiled salad dressing	Very poor
Zygo. spp.	?	Spoiled salad dressing	Very poor

Growth Measurement. Yeast growth was measured by turbidity using a Klett-Summerson photoelectric colorimeter. Blue filter No. 42 was used because it brought most of the readings within the sensitive range of the galvanometer scale. The instrument was adjusted to zero with distilled water, and checked periodically during use. It was found essential to shake all tubes thoroughly directly before making a reading to effect distribution of settled cells. Distribution was checked by making a reading, shaking thoroughly again, and rereading. Throughout these studies 30°C. was used as the incubation temperature.

To test the sensitivity of the method a one-liter batch of Medium A was prepared and inoculated with Jerez No. 5. After incubation for 72 hours, distilled water dilutions were prepared. A comparison of the dilution and instrument reading showed that a straight line relationship existed.

Inoculum. Stock cultures of the organisms were carried on V8 Agar in accordance with Morse and Heaton (1950). For use the organism was inoculated into five ml. of Medium A, and transferred by standard wire loop for three successive days. After thorough shaking, 0.1 ml. of the last transfer was pipetted to 100 ml. of sterile physiological saline solution. After thorough mixing, 0.01 ml. of this dilution was used as the inoculum. This dilute inoculum was used to minimize trace metabolite carry-over.

Inhibitory Level. One of the first relationships to be established was that between benzoate concentration required for growth inhibition and the two strains of yeast selected for use in these studies. Medium A was prepared containing sodium benzoate from 0 up to 0.200 per cent graduated in 0.025 per cent levels. The experiment was repeated five times in triplicate and the average results are presented here (Table 3).

TABLE 3

Inhibitory Effect of Sodium Benzoate on Yeast Growth

Sodium Benzoate, pct.	Incubation time in hours					
	48	72	144	48	72	144
	Jerez No. 5[1]			N.Y.D.[2]		
	Turbidity readings on Klett-Summerson					
0.075	—[3]	—	—	—	—	—
0.050	—	—	47	5	47	81
0.025	93	142	220	25	143	—
Control	295	375	390	259	307	—

[1] *S. ellipsoideus*, Jerez No. 5. [2] *S. cerevisiae*, bakers' (National Yeast Co. Active Dried Strain).
[3] "—" indicates not measurable.

Growth of Jerez No. 5 was inhibited by 0.05 per cent sodium benzoate for 72 hours, but growth appeared, though slight, at 144 hours. The NYD strain showed barely perceptible growth at the 0.05 per cent level at 48 hours, and slight growth at 144 hours. It was considered that for the purpose of these studies, 0.05 per cent sodium benzoate was the inhibitory level for both strains of yeast.

Inoculum Size. It is well established that inoculum size is of great importance in studies of this type. Previous workers have established that a large inoculum produces growth under conditions not conducive for growth from a small inoculum. Suggested reasons include carry-over of nutrients with the inoculum, and in large inoculum population, production of necessary nutrients to provide a "self-starting" reaction. To investigate the relationship between inoculum size and inhibition characteristics, the following experiment, using an incremental inoculum size, was conducted.

After incubating the third transfer for 24 hours at 30°C., the cultures were thoroughly shaken and 1, 2, 3, 4, and 5 ml. portions of plain Medium A and Medium A containing 0.05 per cent sodium benzoate were inoculated with 0.02 and 0.05 ml. of the above dilutions. All tests were run twice in triplicate, and studies were made with both yeasts, Jerez No. 5 and NYD (Table 4).

TABLE 4

Effect of Inoculum Size and Sodium Benzoate on Yeast Growth in Medium A

Medium (ml.)	Jerez No. 5[1]		N.Y.D.[2]	
	Control	Benzoate, 0.05 pct.	Control	Benzoate, 0.05 pct.
	0.02 ml. inoculum			
	Turbidity readings on Klett-Summerson			
1	270	15	282	30
2	275	12	295	46
3	355	130	290	70
4	271	113	302	77
5	290	150	302	81
	0.05 ml. inoculum			
	Turbidity readings on Klett-Summerson			
1	297	80	310	63
2	320	132	310	84
3	355	135	310	84
4	275	147	310	85
5	320	150	310	87

[1] *S. ellipsoideus*, Jerez No. 5. [2] *S. cerevisiae*, bakers' (National Yeast Co. Active Dried Strain).

The studies with Jerez No. 5 showed that with an increasing inoculum in the presence of sodium benzoate more growth appeared. This was not true in tubes containing no benzoate; inoculum size did not affect final growth. In the studies with NYD, inoculum size did not affect final growth in media with and without benzoate.

Benzoate on Actively Fermenting Cultures. A rule of long standing for the use of benzoate as a preservative in foods states that once a food has started to spoil by fermentation the addition of sodium benzoate is of no value. Since no published data were available supporting this view it was decided to test its validity. The medium was tubed in 2.5 ml. amounts and inoculated according to previously described procedures. After 24 hours' incubation, 2.5 ml. of sterile medium, containing sodium benzoate in such concentrations that the dilution gave the desired final concentration, were added. After thorough mixing readings were taken. Benzoate levels studied were from 0 to 0.200 per cent in 0.025 per cent graduations. After incubation readings were again made. The average results of five studies are presented (Table 5). It is apparent that rapid growth ensued in the tubes without benzoate, while growth was heavily restricted

in all tubes containing it. The use of benzoate as a preservative is beneficial even in material which has already begun to ferment.

Before undertaking a detailed study in an attempt to establish the particular nutrient for which sodium benzoate might be acting as an antimetabolite, it was decided to conduct a general study employing yeast extract and liver extract. The thought was that should either of these substances reverse the effect of benzoate upon yeast growth, a more detailed study could be made in an attempt to isolate the particular substance responsible for the reversal. Accordingly two series of tubes containing Medium A were prepared, one plain, and the other containing 0.05 per cent sodium benzoate. Both series were prepared so that they contained final concentrations of Difco Yeast Extract as follows: 0, 25, 50, 100, 250, and 500 p.p.m. Inoculation was then made as described above and the tubes were incubated up to 144 hours. No growth was observed in any of the tubes containing sodium benzoate though good growth was observed in the controls.

TABLE 5

The Effect of Sodium Benzoate on Actively Growing Yeast Cultures

Sodium Benzoate, pct.	Jerez No. 5[1]			N.Y.D.[2]		
	Turbidity readings on Klett-Summerson					
	Initial[3]	24 hr.	Difference	Initial[3]	24 hr.	Difference
Control	187	500	313	184	322	138
0.025	200	244	44	195	218	23
0.050	200	230	30	192	212	20
0.075	192	219	27	188	190	2
0.100	198	230	32	184	185	1
0.125	196	248	52	185	200	15
0.150	190	245	55	185	212	27
0.175	205	230	25	180	210	30
0.200	210	230	20	186	213	27

[1] *S. ellipsoideus*, Jerez No. 5. [2] *S. cerevisiae*, bakers' (National Yeast Co. Active Dried Strain).
[3] Turbidity immediately after addition of benzoate.

This experiment was repeated using Difco Bacto-Liver in place of Yeast Extract. Again no growth occurred in those containing sodium benzoate. Both of these experiments were repeated five times with the same result.

DISCUSSION

The medium presented in these studies, though somewhat complicated to prepare, proved a good growth medium for the two organisms used in these experiments. Evidence of this is apparent from the studies with yeast and liver extract, where the growth in unenriched medium was equal to that containing either extract. The method of using frozen concentrates proved time-saving and convenient.

The two organisms selected for these studies grew profusely on Medium A. Of the organisms considered in the preliminary study all grew well except the two organisms isolated from spoiled salad dressing. It was considered that these organisms were probably best adapted to a medium of different composition, though they grew well on V-8 Agar. No attempt was made to find the growth requirements of these two organisms. Upon reinoculation into a simple salad dressing, both organisms produced spoilage after a long incubation period.

The study in which the inoculum size was changed gave variable results. Both organisms showed much better growth in the tubes without benzoate.

The inocula used in these studies were larger than that used in the benzoate inhibitory level studies, and since measurable growth resulted it is established that inoculum size influences benzoate inhibitory level. The N.Y.D. yeast displayed little difference in the inoculum levels studied. However, the Jerez No. 5 yeast showed significantly greater growth at the higher inoculum levels.

The results of the study of the effect of sodium benzoate on actively growing yeast cultures proved enlightening. Growth continued rapidly in control tubes, whereas in all tubes containing benzoate growth was sharply reduced. Of significance here is the inhibitory effect exhibited at the lowest level tried, 0.025 per cent. This contrasts with the earlier study in which minute inocula showed growth up to 0.050 per cent. Closer examination of the growth phase would be of interest here since it has been previously established that growth phase is important to inhibition characteristics.

The studies employing yeast and liver extract proved the medium to be excellent for the organisms studied. The addition of the extracts induced no additional growth. At the levels studied, the nutrilites, both known and unknown, contained in yeast and liver extracts are incapable of reversing the effect of sodium benzoate.

SUMMARY

A new synthetic medium for yeast nutrilite study is presented. Preparation from frozen concentrates is proposed.

Sodium benzoate (0.05 per cent) was found to inhibit the growth of Jerez No. 5 and National Grain Yeast, Active Dried, in the proposed medium. This inhibitory effect was also exerted upon actively growing yeast cultures. Inoculum size was important to resultant growth of Jerez No. 5 in the presence of 0.05 per cent sodium benzoate. Yeast extract and liver extract when added up to 500 p.p.m. failed to reverse the inhibitory effect of sodium benzoate.

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A BUFFERED BORIC ACID LACTOSE MEDIUM FOR ENRICHMENT AND PRESUMPTIVE IDENTIFICATION OF *ESCHERICHIA COLI*

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INTRODUCTION

The coliform bacteria, collectively, almost always have been used as indicators of the sanitary quality of water and frequently of other foods as well. Consequently, most attention has been given to the development of more refined methods for estimation of total numbers of coliform bacteria without regard for selective isolation or enumeration of individual species. Recently emphasis on sanitary supervision throughout the food industries has greatly stimulated interest in bacteriological quality control, and the sanitary aspects of such control have been particularly zealously considered. As a result, emphasis has sometimes been placed arbitrarily on the most probable numbers of all coliform bacteria rather than the most probable number of particular species of the colon group present in a food.

Bacteria of the genus *Aerobacter* apparently occur on, or in, many foods; quite frequently these types are intermixed with the species *Escherichia freundii* and *Escherichia intermedium*. Although this is apparently not so well known, all of these particular bacteria also grow rampant in food plants on surfaces which are continually in contact with the food product being processed. In most instances these three types may occur to the exclusion of *Escherichia coli*. If the presence of *E. coli per se* is a good indication of possible human or animal contamination, it is obvious that mere total numbers of coliform bacteria may not constitute an equitable or attainable index of the sanitary quality of foods in the strict sense. It is believed that the question of a suitable index of sanitary quality of foods should not be left to an arbitrary choice. Therefore, a method which would permit selective enrichment and isolation of *E. coli* would be advantageous to assist in determination of the significance of the various coliform bacteria found in foods.

Several methods have been described which, by physical or chemical means, selectively favor the growth of *E. coli* and related species. Eijkman (1904), who was among the first to propose such a method, utilized glucose broth at an incubation temperature of 46°C. to differentiate the coliform bacteria found in the feces of warm-blooded animals from those encountered in cold-blooded animals. The Eijkman test was accepted in Germany for some time before Leiter (1929) called attention to its possible value in this country. Now, however, the Eijkman test, as modified by Perry and Hajna (1933, 1935, 1944) and Hajna and Perry (1939, 1943) is rec-

ognized as an aid in the differentiation of *E. coli* from other coliform bacteria. Furthermore, their modified "EC medium" used with an incubation temperature of 45.5° ± 0.2°C. has been proposed as an alternative to isolation and identification of *E. coli* by the usual standard procedure. This high temperature has however been reported to be inhibitory for some strains of *E. coli*.

Chemicals such as boric acid (Levine, 1921), hexamine (Calisti, 1931), and telluric acid (Chalmers, 1934) also have been suggested for use in lactose media to favor the growth of *E. coli* and suppress the activity of the other coliform bacteria. Of these, boric acid probably has received the most attention since Levine first reported that it exerted a greater inhibitory effect on the growth of strains of *Aerobacter aerogenes* than upon cultures of *E. coli*. Confirmation of this observation was reported by Levine, Epstein, and Vaughn (1934).

Additional proof of the usefulness of boric acid lactose broth as a means of distinguishing between *E. coli* and other coliform bacteria was reported by Vaughn and Levine (1935) and Vaughn (1935 a, b) in brief abstracts. In these studies, never reported in full, the effects of concentration of boric acid, nutrients, reaction (pH), buffer mixtures, bile, and variations in temperature of incubation on growth and gas production by coliform bacteria were systematically evaluated. As a result, the medium described in 1934 was modified. With this modified medium incubated at 42.5-43.5°C. (temperature of the medium) practically all of the cultures of *E. coli* produced gas in 48 hours, whereas gas-production was suppressed for all of the isolates of *Aerobacter* and, with few exceptions, the strains of *E. freundii* and *E. intermedium*.

Varied experiences have been reported by other investigators who have used this boric acid medium. Bartram and Black (1936) were the first to confirm, in part at least, the possible usefulness of the medium. More recently Wolford and Berry (1948) have reported the medium to be useful as an aid in the differentiation of *E. coli* from other coliform species. The medium also has been suggested for use as a screening presumptive test for *E. coli* by Beisel and Troy (1949). However, Poe, *et al.* (1936) and Poe and Charkey (1948) have reported that a modification of a boric acid lactose broth medium, with or without bile, is not suitable for differentiating between species of *Escherichia* and *Aerobacter*.

EXPERIMENTAL

The following discussion will be limited to a brief resumé of the development of the boric acid medium described by Vaughn and Levine (1935) together with a description of the results of further studies on the efficacy of this medium for differentiation of pure cultures of coliform bacteria and its application to the direct isolation of *E. coli* from contaminated samples.

DEVELOPMENT OF THE BORIC ACID MEDIUM

Attention had been given to the development of a medium which would consistently favor the growth of *E. coli* and yet selectively inhibit the other coliform species when the boric acid medium was first described in 1934. Thus, the concentrations of boric acid and proteose peptone (Difco) had been selected on the basis of investigations with boric acid resistant cultures representing the recognized coliform species, although the concentrations of the other ingredients had of necessity been an arbitrary choice. It

was believed that other factors might influence the growth of coliform bacteria in the borated medium. Therefore, tests were made to determine the effects of acidity (pH), concentration of buffer mixtures, concentration of bile, and variations in temperature on the growth of coliform bacteria in the presence of boric acid.

The test organisms used were the same in all cases: 10 cultures of *E. coli*, 10 strains of *Aerobacter*, and 5 isolates of *E. freundii*. Inoculations always were made from 24-hour nutrient broth cultures of these strains with a small loop (2 mm. dia.). All of these isolates were able to grow and produce gas at 37°C. in the boric acid medium composed of 10 gm. of proteose peptone (Difco); 5 gm. of lactose; 3 gm. of dibasic potassium phosphate (anhydrous K_2HPO_4); 3.25 gm. of boric acid; and 10 ml. of Andrade indicator per liter of distilled water. This medium was used as the basal medium for further experiments.

All test media were tubed in 7-10 ml. portions in Durham tubes, sterilized at 15 p.s.i.g. steam pressure for 15 minutes, cooled immediately, and incubated overnight at 37°C. before inoculations were made.

Effect of Acidity (pH) of the Medium. It is well known that the initial pH value of the medium may markedly influence the growth of bacteria. The acidity best suited to the growth of the test cultures in the borated lactose medium was determined by growing the cultures in portions of the medium adjusted to pH values of 6.0, 6.5, 7.0, 7.3, 7.5, 7.7, and 8.0 before sterilization. The acidity was adjusted by the addition of requisite quantities of either monobasic, dibasic, or tribasic potassium phosphate which replaced the 3 gm. of dibasic potassium phosphate present in the original basal medium.

The initial pH value of the medium did influence the ability of the test cultures to grow in the boric acid lactose broth. An initial pH value of 7.5 appeared to be most favorable for the evolution of gas by all cultures tested. However, with an initial reaction of pH 7.0, the *Aerobacter* and *E. freundii* isolates showed less gas production than at the higher levels tested. For this reason it was decided that an initial pH value of between 6.8 and 7.2 would probably be most favorable for growth of *E. coli* and selective inhibition of other coliform species by boric acid.

Effect of Bile on the Selectivity of the Medium. Among others, Levine (1922) and Coles and Levine (1927) have shown that bile may stimulate or retard the growth of coliform bacteria, depending upon the concentration of bile in the medium and the reaction of the medium. On the basis of these observations it was hoped that the addition of bile to the boric acid medium might assist in further inhibition of the *Aerobacter* and *E. freundii* strains which were unusually resistant to boric acid.

The effect of the concentration of bile was determined by adding bacto-oxgall (Difco) in concentrations of 1, 2, 3, 4, and 5 per cent, respectively, to portions of the basal boric acid medium which had been adjusted to pH 7.3 by the addition of 5 gm. of dibasic potassium phosphate. The resultant media were tubed, sterilized, inoculated, and incubated as described above.

The results obtained indicated that bile in concentrations of more than 2 per cent in the presence of boric acid may significantly delay growth and gas production in lactose broth by coliform bacteria irrespective of type. Even the presence of 1 per cent bile in the boric acid medium delayed growth of one of the test strains of *E. coli*.

Effect of pH and Bile on Selectivity. For the purpose of determining the initial pH value most satisfactory for the growth of the test organisms in the presence of 2 per cent bile in the boric acid lactose medium, portions of the basal medium were adjusted to reactions of pH 6.0, 6.5, 7.0, 7.3, 7.5, 7.7, and 8.0 before sterilization. The desired reactions were obtained by the addition of KH_2PO_4, K_2HPO_4, or K_3PO_4 in quantities necessary to produce the pH values listed above. All pH values were determined by use of the glass electrode. The resultant media were prepared and inoculated in the usual manner.

Although it was found that a pH value of 7.0 might be desirable to use in the boric acid lactose broth with 2 per cent bile, the addition of bile to the medium was of questionable value. The same inhibition of *E. coli* noted in the previous experiment prevailed in these tests.

Effect of Concentration of Buffer Mixtures on Selectivity of the Medium at 37°C. The previous observations indicated that a reaction ranging between pH 6.8 and 7.2

would be desirable to use with the basal borated medium. It was thought that buffer mixtures concentrated enough to maintain the pH value of the inoculated medium at or near its initial value for some time might be advantageous. Sørensen's KH_2PO_4—$Na_2HPO_4 \cdot 2H_2O$ buffer mixture recalculated in terms of KH_2PO_4—K_2HPO_4 was found to be satisfactory. Buffer mixtures which maintained pH values of 6.8 and 7.0 were used to replace the single phosphate salt previously used to adjust the pH value of the basal medium.

The addition of 10.459 gm. of K_2HPO_4 and 5.446 gm. of KH_2PO_4 per liter was required to maintain the pH value of the inoculated basal medium at or near 6.8. To buffer the inoculated basal medium at pH 7.0 required the addition of 12.203 gm. of K_2HPO_4 and 4.085 gm. of KH_2PO_4 per liter. These concentrations were efficient but lesser amounts of either mixture did not control the pH well.

When inoculated with the test cultures, these two buffered media allowed a greater evolution of gas from lactose by the *E. coli* strains in the presence of boric acid than that produced in the media adjusted to pH values of 6.8 but not highly buffered. However, the buffer mixtures had no discernible effect on further inhibition of the boric acid resistant isolates of *Aerobacter* and *E. freundii* used as test cultures.

The following medium buffered at pH 7.0 was chosen for further investigation:

Proteose peptone (Difco)	10 gm.
Lactose	5 gm.
K_2HPO_4 (anhydrous)	12.203 gm.
KH_2PO_4 (anhydrous)	4.085 gm.
Boric acid	3.25 gm.
Distilled water	1000 ml.

The use of 10 ml. of Andrade's or other suitable indicator was optional. The medium was tubed in Durham tubes, sterilized at 15 p.s.i.g. steam pressure for 15 minutes and cooled immediately after withdrawal from the autoclave. Inoculations were made in the manner already described.

Effect of Temperature of Incubation on Selectivity of the Buffered Boric Acid Lactose Broth. As already stressed, several of the test cultures of *Aerobacter* and *E. freundii* resembled the *E. coli* types very closely in their resistance to boric acid when grown at 37°C. Attempts to suppress the growth of these *Aerobacter* and *E. freundii* cultures in the borated lactose broth by the addition of bile, by varying the acidity (pH) of the medium and by the use of buffer mixtures to control the acidity were not entirely successful. It was hoped that investigation of the effect of the temperature of incubation of the buffered boric acid medium might reveal a means for further selective inhibition of these boric acid resistant strains. Precedent for this hope stemmed from the work of Eijkman (1904), who first revealed that only *E. coli* types would grow at 46°C.

In order to determine the effect of temperature on growth and gas production, the buffered boric acid lactose broth and a control medium (the buffered medium without boric acid) were inoculated in quadruplicate with each of the test cultures previously used. One of each of the 4 sets of 25 inoculated tubes was then incubated at medium temperatures of 37°C., 40-41°C., 42.5-43.5°C., and 45-46°C., respectively. Observations on gas production (Table 1) were recorded at daily intervals.

The higher temperatures of incubation had a striking effect on gas production by the test cultures grown in the buffered boric acid medium. At 45-46°C. none of the test cultures produced gas in the borated medium, even after 4 days, and only the *E. coli* isolates grew and produced gas in the control medium. When the borated medium was incubated at 42.5-43.5°C. all of the *E. coli* cultures produced gas within 2 days whereas none of the *Aerobacter* cultures produced gas until after 2 days, and the cultures of *E. freundii* did not produce gas even after 4 days. At lower temperatures (40-41° and 37°C.) the increase in the number of *Aerobacter* and *E. freundii* which produced gas in the boric acid medium was marked.

As previous studies had indicated that many strains of *E. coli* did not grow or produce gas at 46°C., an incubation temperature of 42.5-43.5°C. appeared to be the most favorable for incubation of the buffered boric acid lactose broth for further testing of the medium.

TABLE 1

Effect of Temperature and Boric Acid on Growth of Coliform Bacteria in a Buffered Lactose Broth[1]

	Control medium (no boric acid)				Buffered boric acid-lactose medium			
Days incubation:	1	2	3	4	1	2	3	4
Test cultures	Number producing gas							
Incubated at 37°C.								
E. coli (10 strains)	10	10	10	10	10	10	10	10
Aerobacter[2] (10 strains)	10	10	10	10	2	5	5	5
E. freundii[3] (5 strains)	5	5	5	5	0	1	1	1
Incubated at 40-41°C.								
E. coli (10 strains)	10	10	10	10	10	10	10	10
Aerobacter[2] (10 strains)	10	10	10	10	1	2	2	2
E. freundii[3] (5 strains)	5	5	5	5	0	0	0	1
Incubated at 42.5-43.5°C.								
E. coli (10 strains)	10	10	10	10	9	10	10	10
Aerobacter[2] (10 strains)	2	5	7	8	0	0	1	2
E. freundii[3] (5 strains)	1	1	1	1	0	0	0	0
Incubated at 45-46°C.								
E. coli[4] (10 strains)	10	10	10	10	0	0	0	0
Aerobacter[2] (10 strains)	0	0	0	0	0	0	0	0
E. freundii[3] (5 strains)	0	0	0	0	0	0	0	0

[1] All temperatures refer to the temperature of the medium. [2] Includes both *A. aerogenes* and *A. cloacae* species. [3] Includes only *E. freundii*. [4] Previous studies had indicated that many strains of *Escherichia coli* would not grow at 46°C. in lactose broth.

Gas Production by Coliform Bacteria in the Buffered Boric Acid Lactose Broth at 42.5-43.5°C. The preceding investigation indicated that the selectivity of the buffered boric acid medium was enhanced by incubation at 42.5-43.5°C. To test this assumption a large number of pure cultures of coliform bacteria were grown in the medium. The medium was first tested in 1934-1935 with 631 pure cultures, comprising 227 strains of *E. coli*, 305 cultures of *Aerobacter*, and 99 strains of the *E. freundii* group isolated from a wide variety of sources. In 1948-1950, 401 cultures including 325 strains of *E. coli*, 56 strains of *Aerobacter*, and 20 isolates of the *E. freundii* group were tested. The 1934-1935 results represent tests made in an air incubator with inoculations from 24-hour nutrient broth cultures. The 1948-1950 tests were made in an oil bath with inoculations from 24-hour nutrient agar slant cultures. A 2-mm. diameter loop was used for all inoculations. For comparison, the results of these two experiments are indicated separately (Table 2).

The buffered boric acid lactose broth, incubated at a temperature of 42.5-43.5°C., markedly inhibited gas production by the *Aerobacter* and *E. freundii* groups, whereas gas production by *E. coli* strains was little affected. The results suggest that the modified boric acid medium may be useful for selective isolation of *E. coli*.

GAS PRODUCTION BY OTHER BACTERIA IN THE BUFFERED BORIC ACID MEDIUM

The previous observations have shown that *E. coli* types are selectively favored in the buffered boric acid lactose broth whereas the other coliform types are inhibited. It was concluded therefore that the medium might be found to be useful as a presumptive test or preliminary enrichment medium for *E. coli*. Many times, however, spore-forming bacteria and yeasts have been associated with spurious presumptive tests for the detection of coliform bacteria in water supplies. Therefore, it was of interest to observe whether the medium would inhibit the micro-organisms which sometimes cause false positive presumptive tests. Accordingly, representative cultures of various lactose-

TABLE 2

Gas Production by Coliform Bacteria in Buffered Boric Acid-Lactose Broth Incubated at 42.5-43.5°C.

Section	Number of cultures	Days of incubation at 42.5-43.5°C.			
		1	2	3	4
		Per cent producing gas			
1935 results[1]					
Escherichia coli	227	92.1	96.5	96.5	96.9
Aerobacter group	305[2]	0.0	0.0	2.6	3.3
Escherichia freundii group	99[3]	4.0	4.0	4.0	6.1
1950 results[4]					
Escherichia coli	325	98.2	98.5	98.5	98.5
Aerobacter group	56	0.0	0.0	0.0	0.0
Escherichia freundii group	20	0.0	0.0	0.0	0.0
Combined results					
Escherichia coli	552	95.7	97.6	97.6	97.8
Aerobacter group	361	0.0	0.0	2.2	2.8
Escherichia freundii group	119	3.4	3.4	3.4	5.0

[1] Air incubator controlled to 43.0 ± 0.5°C. in the medium. [2] Includes both *A. aerogenes* and *A. cloacae*. [3] Includes both *E. freundii* and *E. intermedium*. [4] Oil bath controlled to 43° ± 0.1°C. in the medium.

fermenting bacteria and yeasts, capable of fermenting lactose at 42.5-43.5°C., were tested in the buffered boric acid lactose broth. The bacteria investigated included 14 strains of *Bacillus macerans*; 1 isolate of *Clostridium butyricum*; and 11 thermophilic cultures related to *Clostridium thermosaccharolyticum*. Six of these latter cultures were stenothermal in relation to growth although all 11 isolates had optimum temperatures of 55-60°C. Two strains of *Saccharomyces fragilis* also were tested.

In this experiment the inoculated medium was incubated at 43.0° ± 0.1°C. in an oil bath. The inoculum consisted of a small loopful (2 mm. dia.) of young agar slant cultures of the yeasts and aerobacilli and 0.3 ml. of liver infusion broth cultures when testing the anaerobes.

The results (Table 3) show that the spore-forming bacteria capable of fermenting lactose at 42.5-43.5°C. were not significantly inhibited by boric acid. It is quite apparent therefore that if the buffered boric acid medium is to be used as a presumptive test medium at 42.5-43.5°C. for *E. coli*, a number of unrelated bacteria may cause spurious tests. Nevertheless, the medium used at 42.5-43.5°C. did eliminate additional species which ferment standard lactose at 30-37°C. Among these were included 22 isolates of *Bacillus polymyxa*, 5 strains of the *Clostridium butyricum* type, 2 strains

TABLE 3

Growth of Spore-Forming Bacteria and Yeasts in the Buffered Boric Acid-Lactose Broth

Species	Number tested	Days of incubation at 43° ± 0.2°C.[1]			
		1	2	3	4
		Number producing gas			
Bacillus macerans	14	1	3	11	13
Cl. butyricum	1	1	1	1	1
Cl. thermosaccharolyticum group.					
Stenothermal	6	3	4	4	4
Eurithermal	5	2	3	3	3
Saccharomyces fragilis	2	0	0	0	0

[1] Temperature of the medium.

of *Candida tropicalis*, 2 strains of *Torulopsis kefir*, and one culture each of *Torulopsis sphaerica* and *Zygosaccharomyces lactis*.[1]

PRODUCTIVITY OF THE MEDIUM

The foregoing experiments indicated that the buffered boric acid lactose broth had promise as a presumptive test medium for *E. coli* despite the inhibitory effect of the boric acid. Therefore, it was deemed desirable to investigate the relative productiveness of the medium for *E. coli*. Obviously, the productivity of the boric acid medium, as compared with standard lactose broth, would be a substantial and required test of its suitability as a presumptive test medium.

Growth of Escherichia Coli in the Presence of Aerobacter Cultures. To be entirely useful the buffered boric acid lactose broth must provide continuously favorable conditions for the development of minimum numbers of *E. coli* in the presence of varying populations of other coliform bacteria. This requirement was tested by growing mixtures of varying populations of the two species, *E. coli* and *Aerobacter aerogenes*, in the borated medium held at 43° ± 0.1°C. Two strains of each species were tested: one had been isolated from standard lactose broth; the other recovered from the buffered boric acid broth. The mixtures of the test cultures were prepared from 24-hour broth cultures. The populations of the master cultures were approximated by microscopic counts with the Petroff-Hauser counter. Inocula then were prepared by mixing the desired dilutions of the master cultures in water blanks. The population levels, determined by plating appropriate dilutions in nutrient agar, were based on the average counts of 10 plates. The borated medium (20-ml. portions in 2.5 × 20 cm. test tubes) then was inoculated with 1 ml. of the appropriate mixture and incubated in the oil bath for 48 hours, after which gas production was recorded, and all tubes in each series were streaked on Levine's E.M.B. agar to determine the presence or absence of the two test cultures. Typical results of these productivity experiments are presented here (Table 4).

Under the conditions of these experiments, at least, gas production in the buffered boric acid medium after 48 hours at 43° ± 0.1°C. may be considered as positive evidence of the presence of *E. coli*. Even when the mixture contained a boric acid resistant *Aerobacter aerogenes* strain, gas was not produced unless *E. coli* was found to be present in the culture mixture.

Comparison of Standard Lactose With the Buffered Boric Acid Lactose Broth. Further proof of the possible value of the buffered boric acid lactose medium was sought by investigating its comparative productiveness with that of standard lactose broth for the enrichment of *E. coli per se*. For this purpose 11 samples of Strawberry Creek water, collected at irregular intervals between July and October 1949, were tested.

The two test media were inoculated with one portion each of 10, 1, 0.1, 0.01, and 0.001 ml. of water sample. Each 10-ml. portion was added to 10 ml. of double strength medium. The remaining portions were added to 20 ml. of the single strength broths contained in Durham tubes. The inoculated media then were incubated for 48 hours: the lactose broth at 35°C.; the buffered boric acid medium at 43° ± 0.1°C. E.M.B. agar plates were then streaked from tubes of each of the media containing the smallest portions of the test samples from which gas was produced; and after 24 hours' incubation, typical, well-isolated colonies were transferred to lactose broth. A careful search always was made for coli-like colonies. Then, if gas was formed, the culture was purified, again tested for lactose fermentation and identified with the aid of accepted methods. The results of this experiment (Table 5) indicate that the buffered boric acid lactose broth may be more reliable than standard lactose broth for enrichment and recovery of *E. coli* from contaminated water. Similar results have been obtained with a small number of samples of other foods which have been tested. Lukes (personal communication) has substantiated these observations with a larger number of samples. It is to be stressed, however, that the results obtained are considered indicative, but do not warrant specific conclusions until further substantiated by experiments which are now in progress.

[1] The authors are indebted to Dr. E. M. Mrak for the yeast cultures.

TABLE 4

Gas Production by Mixtures of Aerobacter aerogenes and Escherichia coli in the Buffered Boric Acid-Lactose Broth

Test number	Culture and isolation medium[1]	Cells per ml. of inoculum[2]		Gas production, 48 hours, 43° ± 0.1°C.	Types recovered on Levine's E.M.B. agar
		Coli	Aerogenes		
3	Coli—BA	6	6×10^7	+	Coli and aerogenes equal
		6	6×10^6	+	Coli and aerogenes equal
		6	6×10^5	+	Coli and aerogenes equal
	Aerogenes (boric acid-tolerant)[2] —BA	6	6×10^4	+	Coli and aerogenes equal
		6	6×10^3	—	No coli; all aerogenes
		6	6×10^2	+	Coli and aerogenes equal
		6	6×10^1	+	Coli and aerogenes equal
		6	6	—	No coli; all aerogenes
5	Coli—L	8×10^6	8×10^5	+	All coli; no aerogenes
		8×10^5	8×10^5	+	All coli; no aerogenes
		8×10^4	8×10^5	+	All coli; no aerogenes
	Aerogenes (not boric acid-tolerant)—L	8×10^3	8×10^5	+	All coli; no aerogenes
		8×10^2	8×10^5	+	All coli; no aerogenes
		8×10^1	8×10^5	+	All coli; no aerogenes
		8	8×10^5	+	All coli; no aerogenes
6	Coli—L	6	6×10^7	+	Coli and aerogenes equal
		6	6×10^6	+	Coli and aerogenes equal
		6	6×10^5	+	Coli and aerogenes equal
	Aerogenes (boric acid-tolerant) —BA	6	6×10^4	+	Coli and aerogenes equal
		6	6×10^3	+	Coli and aerogenes equal
		6	6×10^2	+	Coli and aerogenes equal
		6	6×10^1	+	Coli and aerogenes equal
		6	6	+	Coli and aerogenes equal

[1] BA and L indicate whether the test cultures originally were isolated from the boric acid-lactose medium or standard lactose broth. [2] This culture of *Aerobacter aerogenes* grew but did not produce gas in the buffered boric acid-lactose broth in 4 days at 43°C. [3] Divide by 20 for cells per ml. of medium.

TABLE 5

Comparison of Standard Lactose and Buffered Boric Acid-Lactose Broth for Recovery of Escherichia coli

Medium	Standard lactose (48 hours 35°C.)[1]		Buffered boric acid-lactose (48 hours at 43° ± 0.1°C.)[1]	
Sample number	Last gas-positive portion (ml. of sample)	Coliform identification[2]	Last gas-positive portion (ml. of sample)	Coliform identification[2]
1	0.1	*E. coli*[3]	0.1	*E. coli*
2	0.01	*Aerobacter*[4]	0.1	*E. coli*
3	0.01	*E. coli*	0.01	*E. coli*
4	0.01	Spurious	0.1	*E. coli*
5	0.01	*Aerobacter*	0.1	*E. coli*
6	0.001	*E. coli*	0.01	*E. coli*
7	0.001	*E. coli*	0.01	*E. coli*
8	0.001	*E. coli*	0.01	*E. coli*
9	1.0	*E. coli*	1.0	*E. coli*
10	0.01	*E. coli*	0.01	*E. coli*
11	0.01	*Aerobacter*	0.01	*E. coli*

[1] Temperature of the medium. [2] Last gas-positive portion was streaked on Levine's E.M.B. agar; coli-like colonies were selected, purified, and identified by use of the Voges-Proskauer, methyl red, and Koser's citrate tests. All fermented lactose. [3] Selected cultures were V.-P.—, M.R.+, citrate—. (The variety was not determined.) [4] Selected cultures were V.-P.+, M.R.—, citrate+. (The species was not determined.)

DISCUSSION

In conjunction with the development and use of the buffered boric acid medium it is desired to point out that the concentration and kind of peptone (proteose peptone, Difco) and the amount of buffer mixture were first selected because they were found to markedly favor growth of all coliform bacteria. The combined effects of concentration of boric acid and temperature of incubation of the medium were then utilized in order to favor gas production from lactose by *E. coli* and, at the same time, selectively inhibit gas formation or growth of the other coliform bacteria. Thus these two latter factors particularly influenced the usefulness of the medium. Therefore it is mandatory that the concentration of boric acid always be maintained as specified (3.25 gm. per liter of distilled water) and that the temperature of the medium constantly be maintained within the range of 42.5-43.5°C. If these requirements are met conscientiously, the medium is useful both for primary enrichment and rapid presumptive identification of *E. coli*; otherwise it is of little value.

It is recommended, therefore, that an equal volume of double-strength medium be used with all large portions of sample. For example, 10 ml. of double-strength medium should be used with a 10-ml. portion of sample. On the other hand, it has been determined that if at least 20 ml. of single-strength medium is used with portions of 1 ml. or less of sample the efficiency of the medium is not altered.

Furthermore, because accurate control of the temperature of incubation of the medium is such a critical factor, it is recommended that the medium be incubated in an oil bath equipped with an adequate stirring device to insure even distribution of heat. Recent experiences with temperature fluctuations in an ordinary air incubator, a water-jacketed air incubator, and a water bath have re-emphasized the desirability of an oil bath.

SUMMARY

The development and use of the buffered boric acid lactose broth, first suggested by Vaughn and Levine in 1935, has been described in detail.

The selection of ingredients was not an arbitrary choice but based on investigations concerning the effect of concentration of boric acid, nutrients, acidity (pH), and buffer mixtures, together with variation of incubation temperature, on growth and gas production from lactose, by coliform bacteria. A combination of 10 gm. proteose peptone (Difco), 12.203 gm. of dibasic potassium phosphate, 4.085 gm. of monobasic potassium phosphate, 5 gm. of lactose, and 3.25 gm. of boric acid in 1000 ml. of distilled water was found to be most satisfactory for growth of and gas production by *E. coli*. However, maximum inhibition of the boric acid-resistant members of the *Aerobacter* and *E. freundii* groups resulted only when the medium was incubated at a temperature of 42.5-43.5°C. At this temperature the medium was found to be useful for presumptive identification of *E. coli* cultures. Therefore, it is mandatory to carefully control the concentration of boric acid and temperature of the medium.

Results of studies on the productivity of the buffered boric acid-lactose broth suggested that this medium, used at 42.5-43.5°C., might be more

reliable than standard lactose broth for enrichment and isolation of *E. coli per se* from suspected samples of water and other foods.

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PREVENTION OF THE GROWTH OF SUCROSE HYDRATES IN SUCROSE SIRUPS

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Growths with a mold-like appearance (Figure 1) sometimes found in frozen fruit were recently shown to be the crystalline sucrose hydrates (Figures 2 and 3) discovered in a study of the sucrose-water phase diagram [Young and Jones (1949)]. Since the presence of these hydrates in frozen fruit products changes the appearance and texture in an undesirable manner, the present work was undertaken to learn how to prevent their formation. The effects of various added substances on the growth of sucrose hydrates in sucrose solutions have been studied. It is hoped that

FIG. 1. Typical sucrose hydrate spherulites (sucrose hemiheptahydrate, $C_{12}H_{22}O_{11} \cdot 3\frac{1}{2}\ H_2O$).

promising results obtained with sucrose solutions as a model will apply to various frozen fruit products of practical interest. It is realized, however, that confirmatory work is desirable on any particular product before commercial application of these results is made to that product. Brekke and Talburt (1950), in a parallel investigation on the prevention of sucrose hydrate formation in cold-processed frozen fruit spreads, obtained results which are in essential agreement with our observations.

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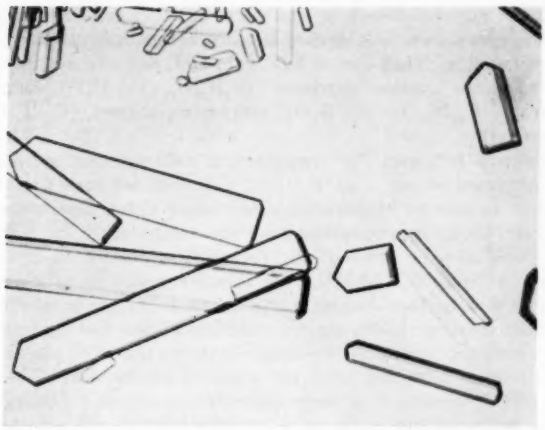

FIG. 2. Sucrose hemipentahydrate (100 ×), ($C_{12}H_{22}O_{11}\cdot 2\frac{1}{2}\ H_2O$).

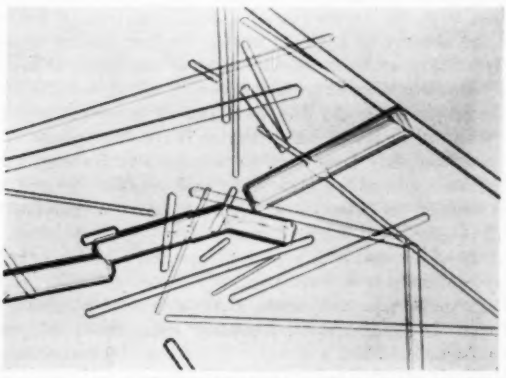

FIG. 3. Sucrose hemiheptahydrate (100 ×) ($C_{12}H_{22}O_{11}\cdot 3\frac{1}{2}\ H_2O$).

In order to indicate the conditions under which sucrose hydrate crystallization may occur, the sucrose-water phase diagram will be briefly discussed before our observations in the present study are presented.

THE SUCROSE-WATER PHASE DIAGRAM

The sucrose-water phase diagram shown in Figure 4 does not include several previously described phases [Young and Jones (1949)], which apparently are not sufficiently stable to be involved in the present problem. Each area in the diagram represents the concentration and temperature range in which the indicated phases may be found at equilibrium conditions (stable or metastable). Thus sucrose hemiheptahydrate

and solution may be found in the area *hdbj*. Each curved line is a solubility curve, shown as a broken line under conditions where the solid phase is metastable. Thus curves *bdh*, *kdg*, *mef*, and *abc* are the solubility curves for sucrose hemiheptahydrate ($C_{12}H_{22}O_{11} \cdot 3\frac{1}{2}\ H_2O$), sucrose hemipentahydrate ($C_{12}H_{22}O_{11} \cdot 2\frac{1}{2}\ H_2O$), anhydrous sucrose ($C_{12}H_{22}O_{11}$), and ice, respectively.

The diagram indicates, for example, that a 60 per cent sucrose solution is undersaturated above +24°F.(−4.2°C.), but becomes supersaturated with respect to sucrose hemiheptahydrate below this temperature. Should this hydrate begin to crystallize and the temperature be lowered, the solution would become more dilute, following curve *db* as crystallization proceeded. At +15°F.(−9.5°C.) the solution would contain only 56 per cent sucrose and further cooling would result in complete solidification of the solution, forming a fine-grained mixture of ice and hydrate crystals called the eutectic. Similarly, the diagram shows that a 30 per cent sucrose solution becomes saturated with ice when cooled to +27°F.(−2.65°C.). Further cooling results in a more concentrated solution (following curve *ab*) as ice is frozen out until, at +15°F.(−9.5°C.), the solution concentration becomes 56 per cent, as before. In the presence of sucrose hydrate seed crystals, further cooling would again result in complete solidification of the solution with the formation of eutectic mixture of ice and sucrose hydrate. In the absence of hydrate nuclei, further cooling would result in further ice formation, and the concentration of the solution would continue to increase, following solubility curve *abc*, until at −22°F.(−30°C.) it would become 80 per cent. At this temperature, however, crystallization is very slow. It should be noted here that ice is the only phase which readily crystallizes spontaneously from sucrose solutions at freezing temperatures.

When hydrate crystallizes from solutions containing over 70 per cent sucrose, it is usually the hemipentahydrate [and its corresponding eutectic below +13°F.(−10.5°C.] which forms.

If a mixture of sucrose hemiheptahydrate and solution containing more than 63.5 per cent sucrose is warmed slowly above +33°F.(+0.4°C.) (*d* in Figure 4), sucrose hemipentahydrate, the stable solid phase between *d* and *e*, should form. In concentrations greater than about 68 per cent, this usually does occur without seeding. These two hydrates are so similar however, that it is of little practical importance which hydrate is present, although only the hemiheptahydrate has been identified in the frozen fruit samples examined.

Sucrose hemipentahydrate, when warmed above +37°F.(+2.8°C.) (*e* in Figure 4), should change to anhydrous sucrose. This change will take place rapidly when anhydrous crystals are present or when the hydrate crystals are crushed, but otherwise the transition may not occur before the crystals melt.

As may be seen from this discussion, the only region in which neither of the two hydrate phases can exist is above and to the left of *abdef*. Unfortunately, this region lies above the region of useful temperatures for freezing preservation. It is also unfortunate that the maximum rate of hydrate formation occurs at about −10°F.(−23°C.) which is close to the temperature used in commercial freezing storage. Although spontaneous

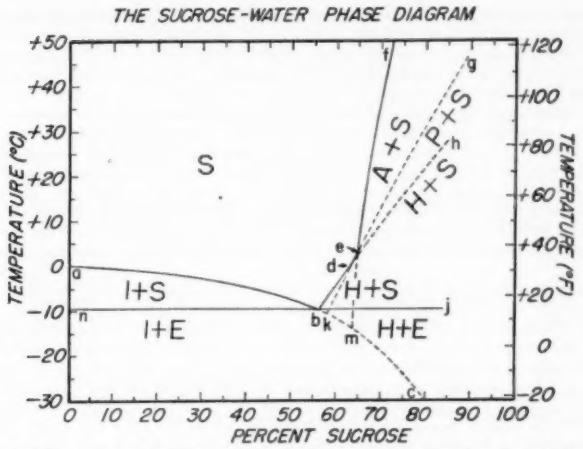

FIG. 4. The sucrose-water phase diagram. S = solution, I = ice, A = $C_{12}H_{22}O_{11}$ (anhydrous). P = $C_{12}H_{22}O_{11}\cdot 2\frac{1}{2}\ H_2O$, H = $C_{12}H_{22}O_{11}\cdot 3\frac{1}{2}\ H_2O$. E = eutectic (a mixture of ice and hydrate).

sucrose hydrate crystallization occurs more readily at high sucrose concentrations, nothing is to be gained by using a low sirup concentration because ice crystallization will rapidly increase the concentration of the remaining sirup to the equilibrium value (curve *abc*, Figure 4) for the temperature used, regardless of the initial concentration. The net result is merely an increase in the amount of ice present and a reduction in the amount of sirup remaining. It should also be pointed out that sucrose hydrate can form from granulated sugar, which will obtain its water of crystallization from the fruit juices.

Two approaches to the problem of preventing sucrose hydrate growth were considered. One was an attempt to find a substance which when added to the extent of a few per cent at most would inhibit the growth of the hydrate by specific surface effects or by marked changes in the viscosity. The other approach was to try to find one or more substances which, when added in higher concentrations, would produce a system which was not supersaturated with respect to crystalline phases other than ice at commonly used freezing storage temperatures. Since no phase equilibrium data were available on which to base such a choice of additives, the investigation was necessarily carried out on an empirical basis.

EXPERIMENTAL

Two sets of survey experiments were carried out. In the first set, sucrose solutions were prepared containing approximately 25, 45, 55, and 60 per cent sucrose and from 0.1 to 20 per cent of the additive. The highest additive concentration used with the 60 per cent sucrose solution was 10 per cent. Concentrations of sucrose and additive are expressed throughout this paper as percentage of the total weight of the final solution contributed by the solids in the additive.

Dextrose (D-glucose), levulose (D-fructose), synthetic invert sugar (a mixture of equal parts of C. P. levulose and dextrose), maltose, low conversion corn sirup (dextrose

equivalent = 29), dextrin (bacteriological grade), glycerine and propylene glycol were used as additives at 1, 5, 10, and 20 per cent; citric acid and ascorbic acid at 1 and 5 per cent, and soluble starch, potato starch, pectin (Pectinum N. F. VII), low methoxyl pectin, gum arabic, gum tragacanth, gelatin, agar-agar, and sodium alginate were used at 0.1 and 1 per cent levels with each sucrose concentration.

Each sample (approximately 10 ml. in a stoppered test tube) was cooled to −10°F. (−23°C.) and seeded with about 0.1 gm. of sucrose hemiheptahydrate on the surface of the solution. It was then stored at this temperature and inspected periodically until the effect of each additive was clearly evident. Additional sucrose solutions containing no additives were seeded and stored along with the others for comparison. The rates of hydrate growth were compared by estimating what part of the sample volume (in tenths) was converted to hydrate. The amount of hydrate was easily estimated in most cases as it grew in one mass from the seed and was whiter than the ice growing in the same sample.

Seeding in this manner does not allow for the possibility that an additive may prevent initial nucleation, but to be effective, an additive must also be able to prevent the growth of sucrose hydrate which is accidentally introduced. In addition, if seeding had not been employed, a much larger number of samples would have been necessary because of the undependability of spontaneous nucleation. The amount of crystalline hydrate seed was purposely made many times the quantity which would be present from accidental seeding, so that the effectiveness of the additives would be clearly shown. The amount of growth occurring in the initial stages in the presence of effective additives is undoubtedly larger in these experiments than if only accidental seeding had occurred.

The temperature, −10°F., was chosen as approximately the optimum for hydrate growth on the basis of previous work with sucrose hydrates.

After the first set of experiments showed that only the sugars offered any promise of preventing hydrate growth, and that higher additive concentrations should be investigated, a second set of solutions was prepared with the following additives: high and medium conversion corn sirup, an enzyme-converted corn sirup (with dextrose equivalents of 61, 43, and 63, respectively) and a mixture of dextrose, maltose, and dextrins in approximately the proportions in which they occur in 43° Baumé corn sirup (22:21:37). Each of these was used at the following concentrations (per cent sucrose: per cent additive): 30:20, 30:30, 35:35, 40:20, and 50:20. Dextrose, levulose, synthetic invert sugar, and maltose were used at 30:20, 30:30, 40:30, and 35:35.[2] In addition, carboxymethyl starches S and W[3] (10 per cent), glycine (5 and 10 per cent), carboxymethyl cellulose (0.25 and 5 per cent), methocel (viscosity = 15 cp) (0.25 and 5 per cent), sodium dihydrogen citrate (5 per cent), and a sodium phosphate buffer (pH approximately 7) (5 per cent) were used in 55 per cent sucrose solutions.

Each solution was divided in most cases into ten portions. One portion was seeded and stored at −10°F.(−23°C.) as before; another portion was seeded and stored at −30°F.(−34°C.). Three tightly stoppered portions were stored at −10°F. and two at −30°F. without seeding. These samples were used to study any behavior of unseeded samples which might not be observed with the seeded samples, such as spontaneous crystallization of the additive or enhanced spontaneous crystallization of sucrose hydrate. The remaining three portions were seeded and stirred well at −10°F. and then warmed to room temperature. Two of these portions were held at room temperature for two hours (during which the hydrate crystals slowly melted) and then were returned to the −10°F. room for storage. The third portion was returned to the −10°F. room after 24 hours at room temperature. The object of seeding at storage temperature, warming, and subsequently cooling again was to determine if the recent temperature history of the sample affected nucleation rate and crystal growth. Such an effect has

[2] The 40:20 and 50:20 samples containing sugar additives were omitted because the results at three months indicated that they were not likely to be effective in these concentrations over longer periods (except maltose which is probably too expensive for commercial use). The ratios used should allow a reasonable prediction of the behavior of these additives at intermediate ratios.

[3] The S and W represent carboxymethyl starches prepared from smooth and wrinkled skin peas, respectively, by Dr. R. M. McCready of this Laboratory.

been reported by Dorsey (1938) in the freezing of pure water. However, the samples subjected to this treatment behaved like the unseeded samples and will be grouped with the latter in discussing the results. Both seeded and unseeded solutions containing 30, 50, and 70 per cent sucrose with no additives were placed at each temperature for comparison. All samples were inspected at intervals as before. After the final inspection, doubtful samples were examined microscopically at +10°F.(−12°C.) in order to determine if the solid present was sucrose hydrate or some other compound.

RESULTS

With nearly 800 samples and 30 additives, a complete tabulation of the results is prohibitively long; the failure of most of the compounds present to retard hydrate growth for even two weeks makes such a tabulation unnecessary.

The compounds which showed promise of preventing hydrate growth after 14 weeks in the first set of experiments are shown in Table 1. All additives not shown in Table 1 gave ratings of 9 to 10 at 14 weeks with the exception of propylene glycol and glycerine. These two additives in 10 and 20 per cent concentrations in 25 per cent sucrose solutions were rated 0. The 20 per cent concentrations in 45 per cent sucrose solutions were rated 1. Both of these additives are unlikely to be used commercially in such high concentrations.

It is apparent from Table 1 that the only additives tried which indicate any reduction of hydrate growth are sugars and dextrin. To simplify the

TABLE 1

Growth of Sucrose Hydrate at −10°F.(−23°C.) After 14 Weeks in Seeded Sucrose Solutions Containing Various Additives (First Set)

Per cent sucrose	25				45				55				60		
Per cent additive	1	5	10	20	1	5	10	20	1	5	10	20	1	5	10
Per cent replacement[1]	4	17	29	44	2	10	18	31	2	8	15	17	2	8	14
Corn sirup, low conversion[2]	6[3]	2	1	0	10	4	2	1	10	10	5	1	10	7	6
Dextrin	10	5	1	1	10	10	2	1	10	10	4	1	10	10	4
Maltose	7	2	0	0	10	6	8	0	10	10	7	2	10	8	5
Levulose	5	3	0	0	10	10	10	3	10	9	8	2	10	9	7
Invert sugar	6	6	4	0	10	10	10	4	10	10	8	2	10	10	8
Dextrose	6	0	1	0	10	10	10	4[4]	10	10	8	3[4]	10	10	9
No additive	9				10				10				10		

[1] The per cent of total soluble solids supplied by the additive.
[2] Dextrose equivalent, 29.
[3] The numbers opposite each additive indicate the volume of hydrate growth in tenths of the total sample volume (0 indicates no growth, 10 complete growth).
[4] Spontaneous hydrate crystallization (spherulites growing without contact with the added seed).

discussion, the word sugar will be used here only for sugars other than sucrose. The results in Table 1 indicate that the additive concentration needed to prevent hydrate growth is determined by the sucrose concentration. For 25 per cent sucrose, 10 per cent of the additive may be effective, but for 55 per cent sucrose, 20 per cent additive is not completely effective. Maltose, dextrin, and low conversion corn sirup appear to give the best results.

Although growth of the hydrate after one month was only about half as great as after three months, the relative amounts of hydrate for the various additives were essentially the same for the two periods.

In sucrose solutions without additives, the rate of sucrose hydrate growth was greatest in the solutions containing 55 per cent sucrose. These solutions showed complete growth in four weeks, while the 45 per cent solutions showed a growth of about 5 and 60 per cent solutions showed a growth of about 6 for the same period.

In the second set of experiments, again only the sugars and corn sirups reduced the rate of hydrate growth, as shown by Table 2 for nine months' storage at −10°F.(−23°C.). In both Tables 1 and 2, any detectable increase in the size of the original seeding material was conservatively given a rating of 1. Thus a rating of 1 in most of the cases shown in Table 2 represents a growth of less than 10 per cent. Maltose, the corn sirups, and the mixture of dextrose, maltose, and dextrin gave the best results; as

TABLE 2

Growth of Sucrose Hydrate at −10°F.(−23°C.) After 9 Months in Seeded Sucrose Solutions Containing Various Additives (Second Set)

Column Number	1	2	3	4	5	6
Per cent sucrose	30	30	35	40	40	50
Per cent additive	20	30	35	20	30	20
Per cent replacement[1]	40	50	50	30	43	29
Corn sirup, high conversion[2]	2[3]	1	1	2	2	5
Corn sirup, medium conversion[2]	1	1	1	2	2	4
Corn sirup, enzyme conversion[2]	2	1	1	2	2	5
Maltose-Dextrose-Dextrin	1	1	1	1	1	1
Maltose	1	1	2	....	1	....
Levulose	6	1	2[5]	....	4	....
Invert sugar	4	1	1	....	4[7]	....
Dextrose	2	5[6]	8[6]	....	6[6]	....
No additive[4]	10	....	....	....	....	10

[1] The per cent of total soluble solids supplied by the additive.
[2] Dextrose equivalents of these corn sirups are: high conversion 61, medium conversion 43, enzyme conversion 63.
[3] The numbers opposite each additive indicate the volume of hydrate growth in tenths of the total sample volume (0 indicates no growth, 10 complete growth).
[4] Seventy per cent sucrose with no additive also gave 10.
[5] Spontaneous hydrate crystallization (spherulites growing without contact with the added seed).
[6] Hydrate growth was partly dextrose monohydrate.

before, levulose and invert sugar were somewhat less satisfactory considering the spontaneous sucrose hydrate crystallization (spherulites growing out of contact with original seed) and the larger amount of growth in the 30:20 and 40:30 concentrations (columns 1 and 5, Table 2).

The seeded solutions containing added sugars other than dextrose showed very little hydrate growth in three months at −10°F. In the seeded solutions containing non-sugar additives, the growth averaged 5 in one month and 9 in three months at −10°F.

No spontaneous crystallization occurred in any of the unseeded samples except those containing dextrose. In a number of these samples containing added dextrose, dextrose hydrate was found by microscopic examination. Although there was very little crystallization of this hydrate during the first month, it was present in almost half of the unseeded samples at the end of three months. After nine months, over half of the unseeded

samples containing added dextrose showed considerable dextrose hydrate growth; six of these samples were rated 10.

At —30°F.(—34°C.), there was almost no growth of sucrose hydrate or dextrose hydrate in nine months in either seeded or unseeded solutions.

DISCUSSION

Our results indicate that the formation of sucrose hydrate in sucrose sirups can be greatly reduced either by lowering the storage temperature to —30°F.(—34°C.) or by substituting one or more other sugars (except dextrose) for a substantial portion of the sucrose.

The use of lower temperatures is very effective even without additives. The samples stored at —30°F. showed practically no hydrate growth even when seeded. This agrees with other observations made during our previous work on sucrose hydrates. Unfortunately, storage at —30°F. is too expensive for commercial use at present. The low rate of hydrate growth at —30°F. is doubtless due to the high viscosity of the sirup. The behavior of sucrose solutions during the phase equilibrium study indicated that the rate of hydrate growth is greatest at about —10°F.(—23°C.). Down to this temperature, increasing supersaturation is the predominating factor; below —10°F., viscosity becomes more important.

The percentage of sucrose which must be replaced by other sugars for satisfactory repression of sucrose hydrate formation depends on the amount of sucrose present as well as on the sugar used as a replacement. In concentrations up to 70 per cent total solids, replacement of 50 per cent of the sucrose by corn sirup or invert sugar reduced the hydrate growth to less than one-tenth of the growth observed when only sucrose was used (Table 2, columns 2 and 3). Since the samples on which Table 2 is based were heavily seeded and hydrate growth is expected to be less in samples which are not intentionally seeded, replacements of less than 50 per cent (except dextrose) giving a rating of 2 in Table 2 should be included in future investigations on the prevention of sucrose hydrate growth in particular frozen food products. Thus the results in columns 4 and 5 of Table 2 indicate that, for some additives, 40 per cent replacement for solutions containing between 60 and 70 per cent solids and 30 per cent for solutions containing less than 60 per cent solids should be considered. In this connection, it is interesting to note that Brekke and Talburt (1950) found very little hydrate for 30 per cent replacement by invert sugar in frozen fruit spreads containing 57 per cent total solids and seeded with sucrose hydrate.

Only the corn sirups and invert sugar are cheap enough for commercial use at present. The three corn sirups mentioned in Table 2 (high, medium, and enzyme conversion) appear to have about the same effectiveness in preventing sucrose hydrate crystallization. Invert sugar is less effective than the corn sirups at about 40 per cent replacement in both 50 and 70 per cent total sugars (Table 2, columns 1 and 5). At 50 per cent replacement with both 60 and 70 per cent total sugars, invert sugar and the corn sirups appear to be about equally effective (Table 2, columns 2 and 3).

The choice of an additive for a particular food product would also depend on its effect on the quality of the product. Several reports have

recently been made on the use of invert sugar and corn sirups in frozen fruit packs [Joslyn (1949), Joslyn, Jones, Lambert, Miller, and Shaw (1949), Sather and Wiegand (1948), Tracy, Sheuring, and Dorsey (1947)].

When high additive concentrations are used, the solubility of an additive component at freezing-storage temperatures may be exceeded and crystallization of this component may then occur. An example of this is the spontaneous growth of crystalline dextrose monohydrate in many of the sucrose solutions to which dextrose had been added. The danger of crystallization of an additive is reduced by replacing part of the sucrose by a mixture of sugars rather than a single sugar, thereby using lower concentrations of each without reducing the total sugar concentration. Of the sugars used as additives in this study, only dextrose showed a tendency to crystallize out. The solubility of dextrose monohydrate at 0°F.(—18°C.), in a 52 per cent sucrose solution has been recorded as 12.7 per cent calculated as anhydrous dextrose [Leighton and Leviton (1935)]. The growth of dextrose hydrate in sucrose solutions containing large amounts of added dextrose was therefore not unexpected. Crystallization of dextrose hydrate in fruit packs containing high percentages of dextrose has been reported by Tracy, Sheuring, and Dorsey (1947) and others. However, the failure of dextrose hydrate to crystallize from solutions containing high concentrations of invert sugar was rather surprising in view of the tendency of levulose to lower the solubility of dextrose in water at +86°F.(+30°C.) [Jackson and Silsbee (1924)]. In this connection, it should be noted that Brekke and Talburt (1950) did observe dextrose hydrate formation in some of their samples containing high concentrations of invert sugar.

Among the factors mentioned by Rabak and Diehl (1944) as influencing the crystallization of sucrose in frozen fruit packs are high sucrose concentration, gradual dehydration during storage and fluctuating storage temperatures. Localized high concentrations may result from evaporation which is accelerated by fluctuating temperature. Spontaneous crystallization is, of course, much more likely to occur when the solution is highly supersaturated. In cases of severe local dehydration, anhydrous sucrose may form on the surface. Our experience with sugars suggests that this anhydrous sucrose may initiate hydrate growth.

Our experience further suggests that contamination of cold rooms with sucrose hydrate may be an important factor in the spread of these hydrates. Such contamination may arise from allowing spilled sirup to dry on the floor or shelves, by exudation of the sirup from containers, or possibly, from opening containers in which dry sucrose hydrate is present. Once a cold room becomes contaminated, sirups exposed to the atmosphere seem to develop sucrose hydrate growths readily.

CONCLUSION

The results of this investigation indicate that the growth of sucrose hydrate in sucrose sirups is repressed by storage at—30°F. or by replacement of a portion of the sucrose by a corn sirup, invert sugar, maltose, or levulose. The frequent spontaneous crystallization of dextrose would prevent its use as a sucrose hydrate inhibitor. As pointed out in the introduction, the effects of any replacement of sucrose on quality as well as on

sucrose hydrate prevention should be investigated on the specific product on which commercial application is desired.

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THERMAL DEATH TIMES OF YEAST IN OIL AND MOVEMENT OF THE YEAST BETWEEN THE OIL AND WATER PHASES OF FRENCH DRESSING [1]

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INTRODUCTION

There have been comparatively few studies of the thermal death times of microorganisms in edible oils. Lang (1934) and Rogacheva (1938) have pointed out, however, that the thermal death times of organisms in fat may be of significance in processing certain foods. They hypothesized that (1) microorganisms might become coated with oil in high-fat foods with the result that heat processes ordinarily considered destructive to spoilage organisms would be inadequate inasmuch as the oil-coated organisms were protected from the lethal effect of moist heat, and (2) that these same organisms might then subsequently be shaken out of the oil into the water phase where growth conditions were more favorable. Zuccaro and Powers (1947) observed spoilage of French dressing which might be explained by the above hypothesis. Yeasts were the spoilage agents. The pH of the product and the fact that it was filled hot appeared to be more than adequate to destroy yeast in aqueous suspension.

REVIEW OF LITERATURE

Microbial spoilage of mayonnaise, salad dressing, French dressing, and related products is usually caused by yeast and aciduric bacteria. Tanner (1944) summarized the work of Bachmann (1916), Pederson (1930), and Charlton, Nelson, and Werkman (1934) on bacterial spoilage of salad dressing and related products. Williams and Mrak (1949) described an outbreak of spoilage in salad dressing caused by *Zygosaccharomyces globiformis*. Fabian and Wethington (1950a, 1950b) also found that *Zygosaccharomyces* organisms were involved in spoilage of salad and French dressings.

Though spoilage does occur, the acetic acid generally holds in check potential spoilage organisms during the normal life of these products, provided the initial contamination is kept low (Joffee, 1942). Anderson, Esselen, and Fellers (1949) and Morse, Fellers, and Levine (1948) have reported the germicidal action of acetic, citric, and lactic acids and reviewed the work of previous investigators. Although there is some disagreement as to the relative bacteriostatic and mycostatic efficiency of these acids, depending upon whether they are evaluated on the basis of percentage acidity or pH level, acetic acid is in general considered to be the most germicidal. It may be expected, therefore, that (1) when lemon juice is used in emulsified foods instead of vinegar, (2) when the acidity is low irrespective of the acidulant used, and (3) when some of the ingredients add appreciably to the microbial load, the acidulant might not inhibit microorganisms completely. A preservative or heat processing may then be required. In practice, French, Russian, Thousand Island dressings, tartar sauce, and sandwich spreads are sometimes filled at elevated temperatures or pasteurized to decrease the microbial load.

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The spoilage organisms in such foods are not ordinarily highly heat resistant. Aref and Cruess (1934) reported that *Saccharomyces ellipsoideus* was killed in grape juice at pH 3.8 in 10 minutes at 57.7°C.(136°F.). Moderate pH variation above or below pH 4.0 did not affect greatly the thermal resistance of the organism. Levine and Fellers (1940) and Joslyn (1948) made similar observations. Etchels and Jones (1948) found that acid-tolerant bacteria were killed more readily in a spiced pickle liquor of low pH than one of high pH. Anderson and Esselen (1948) reported that the thermal death time of *Saccharomyces cerevisiae* in McIlvaine's buffer at pH 3.0 was seven minutes at 60.0°C.(140°F.). At pH 7.0 the thermal death time was 40 per cent greater. Esselen, Kosker, Anderson, and Fellers (1949) found that 10 p.p.m. of allylisothiocyanate (a component of mustard oil) decreased the thermal resistance of *Saccharomyces ellipsoideus* by 50 per cent. Hammer (1938), Beamer and Tanner (1939), and Baker and McClung (1939) have reported the z values of non-spore forming bacteria. Spiegelberg (1940) studied the thermal resistance of both spore and non-spore forming bacteria in acid foods. Anderson, Esselen, and Fellers (1949) and Murdock (1950) have studied the effect of organic acids upon the heat resistance of *Bacillus thermoacidurans*.

Baroni (1911), Dickson, Burke, Beck, and Johnston (1925), and Rodenbeck (1932) observed that organisms coated with oil were less susceptible to heat than those in direct contact with moisture. Lang (1934) found that *Clostridium botulinum* exhibited F values of 2.34, 6.55, 8.00, and 3.66, respectively, in tubes of sardines in brine, mustard sauce, oil, and tomato sauce. Jensen (1942) reported great differences in heat resistance of *Streptococci* in fatty and aqueous media. Yesair, Bohrer, and Cameron (1945) found the equivalent resistance of *Streptococcus* No. 7A (National Canners Association) were 0.002-0.008, 150, and 1500 units, respectively, when heated in broth, moist fat, dry fat, and dry air.

If organisms are temporarily entrapped in the oil phase of high-fat foods, how long can they survive? Tanner (1944) stated that Chiapella (1903) and Guargena (1905) found non-spore forming bacteria and yeast lived from 30 to 50 days in common vegetable oils but that Hall and Van Meter (1916) showed peanut oil to have a germicidal action toward *Escherichia coli*. Wethington and Fabian (1950) reported that *Staphylocci* and *Salmonella* organisms remained viable up to 96 hours in mayonnaise and salad dressing at certain pH levels.

Mudd and Mudd (1924) showed that bacteria pass from water to vegetable oil when the two substances are agitated together. Adding sodium oleate facilitated movement from the water to the oil. Jensen (1942) stated that fatty acids and soaps assist the passage of bacteria into fat. Organisms grown continually in high fat foods passed readily from water into oil.

EXPERIMENTAL PROCEDURE

Throughout the study the yeasts used were grown on vegetable juice agar as described by Wickerham, Flickinger, and Burton (1936) except that the pH was adjusted to 4.8 instead of 6.8. The bacteria were grown on tomato juice agar, described by Tanner (1944) as "Kulp and White's tomato juice agar." Wort agar was also used in parallel with vegetable juice agar for many of the tests. Quicker and better growth was obtained with vegetable juice agar [Morse and Heaton (1950)].

Viability of Acid-Tolerant Organisms in Vegetable Oils. *Saccharomyces cerevisiae, Saccharomyces ellipsoideus, Zygosaccharomyces sp.* (isolated from spoiled French dressing), *Saccharomyces sp.* (isolated from spoiled salad dressing), *Lactobacillus lycopersici*, and *Bacillus thermoacidurans* were inoculated into cottonseed, peanut, and corn oils. The organisms were from cultures 2-3 days old; the inocula consisted of 50 million cells per ml. of oil. Two complete series were run. Duplicate subcultures were made daily, using vigorous agitation during dilution to insure oil removal. Plates were incubated for seven days at 30.0°C.(86.0°F.). Subculturing was continued for 2 weeks beyond the last observable growth to check for "skips."

Thermal Death Time Studies. The four yeasts were grown upon vegetable juice agar in quart fruit jars laid on their side. The yeast crops were harvested after 3-4 days with a sterile spatula, placed onto a sterile filter paper in a Buchner funnel, the funnel

was covered with six layers of sterilized cheesecloth, and air was sucked through the yeast. A 100-watt light bulb was suspended about four inches above the funnel to warm the air slightly. When dry, the yeasts were transferred to sterile test tubes and stored at 7.2°C.(45°F.).

Thermal death time studies were made in cottonseed, corn, peanut, and soybean oils. The pH of the oils were 5.6, 6.8, 6.5, and 6.6, respectively. For the aqueous studies a 0.85 per cent phosphate buffer was adjusted to the same pH levels. After about 75 comparisons had been made, the use of four oils and four buffers was discontinued because no significant differences existed within either series. Thereafter, cottonseed oil and phosphate buffer at pH 5.6 were used. Some studies were also made using cottonseed oil adjusted to pH 4.0 with powdered citric acid, and phosphate buffer at pH 4.0. The pH of the oils was measured by emulsifying the oil with a small portion of water, then measuring the pH of the emulsion.

At 54.4°C.(130°F.) the thermal death times were determined according to the sealed glass tube method of Bigelow and Esty (1920) using four tubes at each time and temperature. Complete destruction was used as the endpoint. The heating lag in oil was 3.50 minutes and in phosphate buffer 3.25 minutes. The constant temperature, heating bath was thermostatically controlled to within 0.3°F.

Because the thermal death times were so short above 54.4°C.(130°F.), two different techniques were used in that range. In the phosphate buffer determinations were made at 60.0°C.(140°F.) and at 71.1°C.(160°F.). At 76.6°C.(170°F.) destruction was too rapid to measure. Test tubes of buffer were submersed in the constant temperature bath until the contents of the tube were at the bath temperature as judged by a thermometer in a similar tube. A loopful of dried yeast was placed in the buffer and agitated. At the end of each time interval a one-ml. aliquot was withdrawn and immediately pipetted into a cold dilution bottle. The tubes were plugged with cotton between each withdrawal. The concentration of organisms in the tube was 166,000,000 (±10 per cent) per ml. as estimated by plate count.

Efforts to disperse the yeast uniformly in oil were unsuccessful. Grinding in a mortar or using a Waring Blendor reduced the viable count below 166,000,000 organisms per ml. No dispersant could be thought of which might not influence the thermal death times. The following system was therefore followed: A loopful of yeast was placed in the oil and left there for the desired length of time. The yeast did not disperse. The loop was then withdrawn and agitated in cool dilution water to dislodge the yeast. After shaking to distribute the yeast, plate counts were made.

With each of the three methods, after the thermal death times had been tentatively established, a minimum of nine replicate determinations were made at each temperature at five time periods above and below the expected survival-destruction line.

Migration of Yeast From Water to Oil and Vice Versa. Known viable yeast cells were placed in sterile water. This inoculum was mixed 1:1 with sterile oil, then agitated in a Waring Blendor. After settling, both phases were examined microscopically for yeast.

To demonstrate movement in the opposite direction, active dried yeast (Fleishmann) was pulverized in a mortar with sterile oil. The inoculated oil was blended 1:1 with sterile water in a Waring Blendor. Again, both phases were examined for yeast after separation. To learn also whether transfer would occur without agitation and whether the yeasts were still viable, one ml. of the inoculated oil was pipetted onto vegetable juice agar plates.

Pasteurization Based Upon Thermal Destruction in Aqueous Phase. A French dressing was made using a typical recipe. Lemon juice was the acidulant, and the pH was 4.0. After preparation, the dressing was sterilized for 15 minutes at 121.1°C.(250°F.). It was then inoculated with dried baker's yeast and agitated in a sterile Waring Blendor. The dressing was filled into thermal death tubes and pasteurized at 60.0°C. (140°F.) for 10 minutes. Calculation from previous data obtained with phosphate buffer and from the heating and cooling lags of the tubes showed that this process was over twice as long as the heating needed to destroy the organisms in the aqueous phase. After pasteurization 12 tubes were incubated directly and 12 were subcultured on vegetable juice agar. An equal number of tubes of sterilized French dressing served as controls. Positive controls consisting of inoculated, but unpasteurized, French dressing were also run.

RESULTS

The longevity tests using cottonseed, peanut, and corn oils showed that the type of oil did not influence the survival period of the organisms. The maximum survival period for *Saccharomyces cerevisiae, Zygosaccharomyces sp., Saccharomyces ellipsoideus, Saccharomyces sp.,* and *Lactobacillus lycopersici* were, respectively, 16, 29, 31, 38, and 29 days. *Bacillus thermoacidurans* was still viable when the tests were discontinued after 85 days. In all studies agreement between replicates was within 2 days.

Thermal Death Times. The thermal death times of the organisms were not significantly different in any of the oils although the pH varied from 5.6 to 6.6. Similarly, no differences were observed in phosphate buffer between pH 5.6 to 6.6. The thermal death times of the yeast are shown in Table 1. Note in Figure 1 that the thermal death times of *Saccharomyces sp.* were the same in cottonseed oil at pH 5.6 and at 4.0. Lowering the pH of the phosphate buffer from 5.6 to 4.0, however, reduced the thermal resistance of the yeast by approximately 50 per cent. The thermal resistance of each organism in oil was much greater than in buffer.

TABLE 1

Thermal Death Times of Four Yeasts in Cottonseed Oil and in Phosphate Buffer at pH 5.6 and One at pH 4.0

Temperature of exposure	(°F.) (°C.)		120 48.8	130 54.4	140 60.0	160 71.1	170 76.6	180 82.2
Organism	Substrate	pH	Time in minutes for complete destruction (166,000,000 cells per ml.)					
Saccharomyces sp.	Buffer	4.0	.....	40.00	3.00	0.13	.....	.....
	Oil	4.0	.....	.....	90.00	3.50	1.25	0.43
	Buffer	5.6	.....	80.00	7.00	0.30	.....	.....
	Oil	5.6	>540	>420	90.00	3.50	1.25	0.43
Saccharomyces ellipsoideus	Buffer	5.6	.....	95.00	6.00	0.33	.....	.....
	Oil	5.6	>540	>420	70.00	2.50	1.17	0.40
Saccharomyces cerevisiae	Buffer	5.6	.....	75.00	5.00	0.25	.....	.....
	Oil	5.6	>540	>420	65.00	2.00	1.00	0.33
Zygosaccharomyces sp.	Buffer	5.6	.....	95.00	7.00	0.33	.....	.....
	Oil	5.6	>540	>420	70.00	2.17	1.08	0.38

Plotting of the thermal death times against temperature did not give straight lines on semilogarithmic paper. However, in the higher temperature ranges the curve appeared to be a straight line. The z values of the yeast in oil were greater than those in buffer as may be seen in Table 2. Inasmuch as no standard exists for setting F values for low-heat resistant organisms, F values were considered to be at 71.11°C.(160°F.) and are designated F_{160}.

The degree of variation encountered in determining the survival-destruction points were moderate. In oil, the differences in time between points always showing destruction and always showing survival averaged 13.4 per cent. In buffer, the average was 13.8 per cent. Wider variations were encountered in the buffer although the average variation was nearly the same.

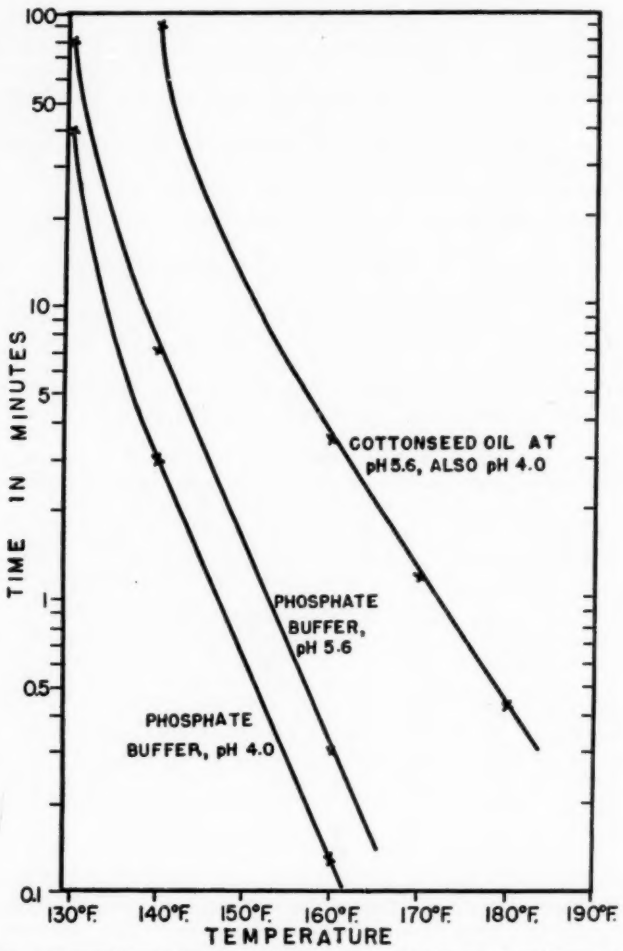

FIG. 1. Thermal death time of *Saccharomyces sp.* in buffer and in cottonseed oil.

Movement Between Phases of an Emulsion. The studies to demonstrate passage of the yeast from one phase to the other showed that emulsification does drive organisms from the water phase into the oil phase. By microscopic estimation about 20 per cent of the yeast were in the oil phase; many organisms were clumped at the interface. Agitation also caused the yeast to move from the oil to the water phase. When the yeasts in oil were pipetted onto vegetable juice agar, they apparently collected at the interface because growth was secured eventually on all plates.

TABLE 2

F_{250} and z Values of Four Yeasts in Oil and Phosphate Buffer at pH 5.6 and One Yeast at pH 4.0

Organism	Substrate	pH	F_{250}	z (in °F.)
Saccharomyces sp.	Buffer	4.0	0.13	14.8
	Oil	4.0	3.50	22.8
	Buffer	5.6	0.30	14.6
	Oil	5.6	3.50	22.8
Saccharomyces ellipsoideus	Buffer	5.6	0.33	16.2
	Oil	5.6	2.50	21.4
Saccharomyces cerevisiae	Buffer	5.6	0.25	15.3
	Oil	5.6	2.00	20.9
Zygosaccharomyces sp.	Buffer	5.6	0.33	15.5
	Oil	5.6	2.17	21.8

The pasteurization experiment showed that yeast in French dressing would survive a process over twice as severe as calculated to be lethal for the organisms in the aqueous phase but sub-lethal for those in the oil phase. The 12 tubes which were subcultured showed viable organisms. The 12 tubes which were incubated directly were slow in spoiling; no spoilage occurred in less than three weeks. None of the sterilized French dressing spoiled, and all of the inoculated, non-pasteurized dressing spoiled.

DISCUSSION

The longevity tests were in accord with the results of previous workers.

The method of determining the thermal resistance of the organisms in oil, though different from customary techniques, appeared to be satisfactory. Reasons can be advanced for considering that the technique might lead to overestimation and underestimation of thermal resistance. Our tests showed that far less variation was encountered in thermal death times when the loopful of yeast was removed from the oil than when efforts were made to disperse the yeast in oil and subsequently remove a representative aliquot by means of a pipette. Had the yeast been thoroughly desiccated, distribution in the oil would have been easier but under ordinary conditions in food the yeast would not be desiccated. The fact that the thermal death times at 54.4°C.(130°F.) in oil and buffer, which were conducted in the standard manner, are in line with the differences existing at higher temperatures suggests that the two techniques were reasonably comparable. In any event, the results are indicative of the relationship that exists between the thermal death times in oil and buffer as the experiment with French dressing proved.

The fact that the thermal death times did not yield straight lines on semi-logarithmic paper is not surprising. According to Ball (1943), this is not unusual. Curves extending over a wide temperature range may not be straight lines. Because the range of temperature studied in the buffer extended from close to the maximum growth temperature of the organisms

to a highly lethal temperature (48.9°C.-71.1°C.), failure of the thermal death times to yield a straight line curve would be expected. Attempts were made to determine the thermal death times in buffer at 76.6°C. (170°F.), but destruction was always encountered at the shortest time interval possible, 2 seconds.

The pasteurization study demonstrated that spoilage could occur through temporary protection of microorganisms from the lethal effect of moist heat when fat enveloped the yeast. In view of the report of Esselen *et al.* (1949) that allylisothiocyanate reduced the thermal death times of *Saccharomyces ellipsoideus* by 50 per cent (the French dressing contained mustard), the fact that spoilage occurred when the process was more than twice as severe than that required to kill the organisms in the aqueous phase suggests strongly that (1) yeasts were in the fat and thus protected from moist heat and (2) with only moderate handling organisms were shaken out of the fat and caused spoilage.

The results are not presented with the recommendation that French dressing and similar products be pasteurized sufficiently to destroy spoilage organisms in the oil phase. For most purposes, it would be better to replace heavily contaminated ingredients or sterilize them prior to mixing. The data do point out a mode of delayed spoilage and possibly indicate that high-short pasteurization might sometimes be desirable for the more liquid products.

SUMMARY

1. A yeast, identified as a *Saccharomyces,* was isolated from a commercially packed, spoiled salad dressing and another yeast, identified as a *Zygosaccharomyces,* was isolated from spoiled French dressing.

2. The thermal death times of these yeasts and of *Saccharomyces ellipsoideus,* and *Saccharomyces cerevisiae* were determined in phosphate buffer at pH 5.6. All were killed in approximately 0.3 minutes at 160°F. (71.1°C.).

3. At pH 4.0, the thermal death time of *Saccharomyces sp.* was reduced to 0.13 minutes at 160°F.(71.1°C.).

4. At either pH 5.6 or 4.0 the thermal resistance of the yeast in oil was higher than in buffer. In oil the organisms could withstand 2.0 to 3.5 minutes at 160°F.(71.1°C.).

5. The z values of the four organisms in phosphate buffer ranged from 14.8 to 16.2; in oil the z values ranged from 20.9 to 22.8.

6. Pasteurization of French dressing, with a heat treatment in excess of that calculated to kill yeast in the aqueous phase, was inadequate for yeast in the oil phase as judged by the fact that spoilage occurred in every case.

7. The theory that relatively low heat resistant bacteria or yeast when coated with oil might survive processes ordinarily considered lethal in aqueous media was demonstrated to be true. Furthermore, it was shown that the yeast might pass from an oil phase to a water phase where, if growth conditions were more favorable, they could produce delayed spoilage of a food which was apparently adequately heat processed.

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ANTIOXIDANT EFFECT OF SOY AND COTTONSEED FLOURS IN RAW PASTRY MIXES AND BAKED PASTRY[1]

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Soy and cottonseed flours, though recognized as being concentrated sources of high quality protein, and manufactured in forms suitable for human consumption, have not been popular as foods. Yet their high nutritive value, coupled with the fact that they can be produced in abundance indicates that they may become food materials of importance. The acceptance of the flours would be determined largely by their effect on the palatability of foods in which they are used.

The use of soy and cottonseed flour as wheat flour supplements has been suggested as a practical means for improving the nutritive value of many diets, Jones and Divine (1942). Small soy-flour additions to bread have been shown to delay staling, Steller and Bailey (1938) and such low-soy bread has been well accepted over a period of time, Soya Food Research Council (1944). Soy flour has been found to delay rancidity in frozen pastry, Overman (1947), and in ration biscuits, Lips, Marshall, White, and Grant (1949).

No work has been reported on the organoleptic properties of foods containing cottonseed flour.

This study was undertaken primarily to determine whether cottonseed flour is comparable to soy flour as an inhibitor of fat rancidity in raw food mixes and in baked goods, and to compare some organoleptic qualities of soy and cottonseed flours in a bland, wheat-flour product. Since a number of different lards as well as two commercial shortenings were used, information was obtained on the relative keeping quality of different fats in prepared foods.

EXPERIMENTAL PROCEDURE

The development of organoleptic rancidity was determined in raw pastry mixes and in baked pastry made with wheat flour only and wheat flour plus cottonseed or soy flour. Unbleached wheat flour, partially defatted cottonseed flour, and low-fat soy flour were used. Seven lards from hogs receiving different protein supplements were used in the wheat flour pastries and mixes. The lard having average keeping quality in the wheat flour series, lard No. 2, was used for comparing the effect of different levels of cottonseed flour on the development of rancidity. Cottonseed flour was substituted for wheat flour at 1, 3, and 10 per cent levels. Five of the lards were used in comparing the effect on keeping quality of 3 per cent cottonseed flour and 10 per cent soy flour substitutions. The mixes and baked pastries were stored in glass jars and incubated at 50°C. until a distinctly rancid odor was noted.

Pastries made with 3 and 10 per cent cottonseed flour, and 10 per cent soy flour substitutions were submitted to a panel of judges for organoleptic evaluation. In addition to comments on flavor, color, and texture, judges were asked to express opinions that would suggest possible best uses for these flours.

RESULTS

Rancidity Development

Raw Mixes. Rancidity developed at different times in the wheat flour mixes made with different lards as shown in Table 1 and Figure 1.

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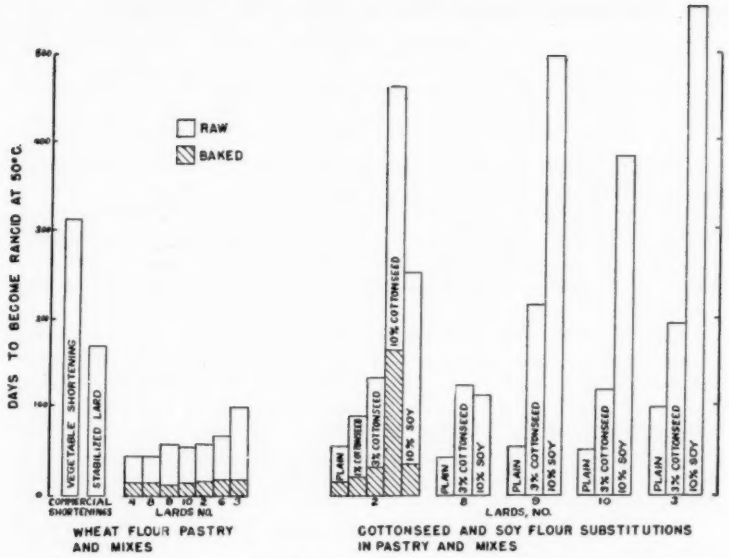

FIG. 1. Development of organoleptic rancidity in baked pastry and raw mixes.

TABLE 1

Number of Days for Development of Organoleptic Rancidity in Baked Pastry and Raw Pastry Mixes, Stored at 50°C.

Lard			Mixes					Baked pastry				
Identification	Peroxide number	Iodine number	Wheat flour	Cottonseed flour			Soy flour	Wheat flour	Cottonseed flour			Soy flour
			100 pct.	1 pct.	3 pct.	10 pct.	10 pct.	100 pct.	1 pct.	3 pct.	10 pct.	10 pct.
No. 8	23.6	59.0	41	....	124	....	112	11	....	....	....	....
No. 9	8.9	59.4	52	....	231	....	497	10	....	....	....	....
No. 10	3.9	60.3	51	....	124	....	385	11	....	....	....	....
No. 2	6.4	56.6	53	89	129	463	249	12	19	27	160	33
No. 3	3.2	57.1	100	....	196	....	562	15	....	....	....	....
No. 4	9.3	66.8	41	....	....	....	....	11	....	....	....	....
No. 6	1.8	55.7	64	....	....	....	....	15	....	....	....	....
Average	....	....	57	89	161	463	361	12	19	27	160	33

The lards having very low peroxide numbers or low iodine numbers might be expected to have better keeping quality in prepared mixes than lards having higher peroxide numbers or high iodine numbers. Lard No. 8, with a comparatively high peroxide number of 23.6, and lard No. 4, with the lower peroxide number of 9.3 but a much higher iodine number (66.8 as compared with 59.0), yielded mixes with the lowest stability. Mixes made with these two lards became rancid in 41 days. Lard No. 3, which

yielded a mix having the best keeping quality, 100 days, had a very low peroxide number and a low iodine number.

A low peroxide number and low iodine number of the lard only partially explain differences in time required for rancidity to develop. Lard No. 6 was similar to lard No. 3 in peroxide number and iodine number, yet the mix made with lard No. 6 did not keep as long as that made with lard No. 3.

Since all lards were from hogs receiving different rations, it is possible that some effect on keeping quality was due to different natural antioxidants present in the lard. No tests for antioxidants were made in this study.

Mixes made with different lards and wheat flour only became rancid in a shorter time than those made with the same lards but containing soy or cottonseed flour. The average length of time required for the development of a rancid odor was 57 days for wheat flour mixes, 161 days for mixes containing 3 per cent cottonseed flour, and 361 days for mixes containing 10 per cent soy flour.

The stability of the lard mixes containing these percentages of soy and cottonseed flour compared favorably with the keeping quality of mixes made with two commercial shortenings. A mix made with a hydrogenated vegetable shortening kept for 313 days before developing an objectionable odor. A mix made with a partially hydrogenated, stabilized lard kept for 170 days before becoming rancid.

When cottonseed flour was substituted for wheat flour at 1, 3, and 10 per cent levels, the development of rancid odor was delayed in approximate proportion to the amount of cottonseed flour used.

Baked Pastries. Baked wheat flour pastries made with the different lards developed rancidity much faster than the raw mixes. They averaged 12 days for development of a rancid odor. Five of the seven baked pastries became rancid within 10 to 12 days. Pastries made with lards No. 3 and No. 6 kept for 15 days before becoming rancid. These two lards yielded the longest keeping raw mixes as well as the longest keeping baked pastry.

Some of the antioxidant effect of the cottonseed flour noted in the raw mixes was retained by the baked pastry. Thus, baked pastry containing 3 per cent cottonseed flour kept more than twice as long as baked pastry made with wheat flour only. Baked pastry containing 10 per cent cottonseed flour kept more than ten times as long as plain wheat flour pastry.

The baked pastry containing 10 per cent soy flour kept a little longer than that containing 3 per cent cottonseed flour, namely, for 33 days as compared with 27 days. The substitutions of soy or cottonseed flour for wheat flour had no significant effect on the breaking strength of the pastry.

Palatability Judging

Judges' opinions were secured on the flavor, color, and texture, and probable satisfactory uses of pastries made with 10 per cent soy flour and cottonseed flour at 3 and 10 per cent levels. Their opinions are given in Table 2.

Judges objected to the color of the 10 per cent cottonseed flour pastry. The products suggested as probable satisfactory uses were, therefore, all dark in color. It should be mentioned that no work was done with these products, so whether molasses or chocolate would mask the greenish-brown color of the cottonseed flour is not known.

TABLE 2

Palatability Judgments of Pastries Made With 10 Per Cent Soy Flour and 3 and 10 Per Cent Cottonseed Flour

Factor	Kind of pastry		
	Cottonseed flour, 3 per cent	Cottonseed flour, 10 per cent	Soy flour, 10 per cent
Color	Not pleasing; brown was dull, not golden.	Too dark for pastry. Too brown. Color not pleasing; greenish-brown rather than golden brown	Very attractive color.
Flavor	Good, rich and pleasing. No unusual flavor.	Flavor somewhat "flat," not as good as the other pastries.	Very good, pleasing. Slight nutlike or bitter, tang characteristic of soy.
Texture	Slightly less crisp than soy or plain.	Less tender and less flaky than plain pastry. Somewhat granular feel.	Crisp and tender.
Probable satisfactory uses	Pastry.	Molasses cookies, gingerbread, brown steamed bread or chocolate or spice products.	Pastry.

SUMMARY

1. Soy flour at the 10 per cent level, and cottonseed flour at 1, 3, and 10 per cent levels were compared for antioxidant value in baked pastry and raw mixes stored at 50°C. Pastry containing 10 per cent soy flour and 3 and 10 per cent cottonseed flour were judged for certain organoleptic qualities.

2. Both soy and cottonseed flour were effective in delaying the development of organoleptic rancidity in raw mixes, and to a lesser extent in baked pastry. Pastry containing 10 per cent soy flour was judged to be very good in flavor, color, and texture. The 3 per cent cottonseed product was satisfactory in flavor and texture, but the color was a dull, greenish-brown rather than a golden brown. Pastry made with 10 per cent cottonseed flour was poor in color and somewhat unsatisfactory in flavor and texture. The substitutions of soy or cottonseed flour for wheat flour had no significant effect on the breaking strength of the pastry.

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PASTEURIZED FROZEN WHOLE EGG AND YOLK FOR MAYONNAISE PRODUCTION[1]

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Pasteurization of liquid egg products greatly reduces the standard plate count bacteria, destroys possible pathogenic bacteria, and prolongs the keeping time of the products according to McFarlane, Watson, and Goresline (1945), Winter, Stewart, McFarlane, and Solowey (1946), and Gibbons, Fulton, and Reid (1946). Freezing and storage of liquid egg products also reduce the viable bacterial count, but not to such an extent as pasteurization as shown by Schneiter, Bartram, and Lepper (1943) and Wrinkle and Winter (1950).

Pasteurization and freezing impair the value of egg products only slightly, if at all, for making angel and sponge cakes and custards according to Miller and Vail (1943), Hanson, Lowe, and Stewart (1947), Slosberg, Hanson, Stewart, and Lowe (1948), and Miller and Winter (1950).

The purpose of this investigation has been to obtain information on the effects of pasteurization and freezing of whole egg and yolk on their emulsifying properties and the stability of their emulsions when used in the production of mayonnaise.

EXPERIMENTAL PROCEDURE

Egg Samples. Grade A shell eggs from the University flock were divided at random into three groups. Group one eggs were dipped at room temperature in an oil used for shell treating eggs. They were stored two to three weeks in a household refrigerator at 40 to 45°F.(4.4 to 7.2°C.) until used. They served as the unfrozen egg control. They were broken out when ready for use and forced through a hand homogenizer, the same as group two eggs.

Group two eggs were broken out, forced through a hand homogenizer, and divided into two lots. Lot one was packaged in pint freezer tubs, frozen at −20°F.(−29°C.) and stored at 0°F.(−17.8°C.) for two to three weeks until used. The other lot was pasteurized according to the method used by Winter, Greco, and Stewart (1946) for four minutes, part at 140 to 142°F.(60.0 to 61.1°C.), part at 142 to 144°F.(61.1 to 62.2°C.), and the remainder at 144 to 146°F.(62.2 to 63.3°C.). The pasteurized samples were then packaged, frozen, and stored the same as the unpasteurized lot.

Group three eggs were broken out and the whites and yolks separated. The yolk was homogenized, separated into two lots and treated the same as the whole egg.

Bacterial Analysis. Standard plate bacterial counts were made according to the method of the Association of Official Agricultural Chemists (1945). Coliform counts were made on desoxycholate agar according to the method used by Leifson (1935).

[1] This study was made possible by a grant from the Refrigeration Research Foundation to the Ohio State University Research Foundation.

Mayonnaise. A mayonnaise formula somewhat similar to the one listed by Lowe (1943) was used. It consisted of:

Whole egg	40 grams
Cider vinegar	30 milliliters
Salt	1 teaspoonful
Sugar	1 teaspoonful
Dry mustard	½ teaspoonful
Corn oil	216 grams

All ingredients were at room temperature when used. The egg, dry ingredients, and 10 ml. of the vinegar were placed in the bowl of a Sunbeam Mixmaster beater with revolving platform. The beating was started at number 4 speed. The emulsification and addition of ingredients were carried out as follows:

	minutes
Mixed egg and dry ingredients	0.5
Addition of 40 ml. of oil from burette	6.0
Addition of 10 ml. of vinegar	0.5
Mixed in the vinegar (no oil)	0.5
Complete rest	1.0
Addition of remainder of oil	6.0
Addition of 10 ml. of vinegar	1.0
Mixed at speed number 1	0.5

The total mixing time was 15 minutes plus one minute of rest. The rotation of the bowl was started as soon as the mixer was set in motion. Oil was added above the surface of the emulsion with the tip of the burette adjusted to three inches above the surface and held constant.

The emulsions were poured into eight-ounce jelly glasses, sealed, and stored at room temperature. Lot 1, prepared with whole egg, was tested after four weeks and both series in Lot 2, prepared with yolk, were tested after two weeks.

When mayonnaise was made with yolk it was substituted for whole egg gram for gram in the formula. The mayonnaise made with defrosted plain yolk was too stiff. The test was repeated with yolk thinned with 15 and 37 per cent water.

For this, 225 gm. of frozen yolk were placed in the mixer bowl and beaten at a speed of 1 for three minutes while 45 ml. of distilled water were added. Thus 40 gm. of the thinned yolk represented 34 gm. of yolk while 30 gm. plus 10 ml. of distilled water represented 25.5 gm. of yolk. By thinning the yolk instead of merely reducing the amount, the percentage of corn oil in the finished emulsions remained constant. In order to offset variations in the finished emulsions due to thixotropy, the unfrozen yolks were also beaten three minutes at speed 1 before use.

Tests. Comparative stiffness of the samples was determined by penetration of a penetrometer cone, carrying a given weight, in 15 seconds.

Comparative stability of the emulsions was determined by weighing duplicate 10 gm. samples, with a minimum of stirring into graduated 10 ml. centrifuge tubes, and centrifuging at top speed in a clinical centrifuge for 15 minutes. The amount of liquid that separated was taken as an indication of instability.

The mayonnaises prepared with yolk were judged for desirable stiffness and flavor by six trained judges.

RESULTS

Destruction of Bacteria. The data are summarized in Table 1. The original counts were high but not beyond the range sometimes found in commercial samples. The high counts were probably due to the fact that the samples were held overnight in a household refrigerator at 40 to 45°F.(4.4 to 7.2°C.) before they were sampled for bacterial analysis and pasteurized.

Pasteurization of the whole egg sample at 140 to 142°F. for four minutes killed more than 99 per cent of the standard plate and coliform

bacteria present. This pasteurization temperature and time killed all the bacteria in the sample of yolk.

Freezing the lot of egg yolk at —20°F. and storing at 0°F. for two to three weeks killed more than 95 per cent of the standard plate count bacteria and all of the coliforms (Table 1).

Mayonnaise Quality. The comparative stiffness of the mayonnaise samples, made with whole egg and judged by penetrometer readings is shown in Table 2. The unpasteurized unfrozen egg produced the stiffest may-

TABLE 1

Influence of Pasteurization and Freezing on the Bacterial Content of Whole Egg and Yolk

Sample and treatment	Bacteria per gm.	
	Standard plate count	Coliforms
Whole egg		
Before pasteurization	7,470,000	73,800
Pasteurized 4 min. at 140-142°F. (60.0-61.1°C.)	4,360	16
Pasteurized 4 min. at 142-144°F. (61.1-62.2°C.)	1,170	33
Pasteurized 4 min. at 144-146°F. (62.2-63.3°C.)	290	7
Egg yolk—unpasteurized		
Before freezing	6,113,000	32,700
After freezing and defrosting	155,000	0
Egg yolk—pasteurized		
Before pasteurization	6,113,000	32,700
Pasteurized 4 min. at 142-144°F. (61.1-62.2°C.)	0	0
After pasteurizing, freezing, and defrosting	0	0

TABLE 2

Stiffness of Whole Egg Mayonnaise Samples (Penetrometer Readings)[1]

Replications	Unpasteurized samples		Pasteurized frozen samples		
	Unfrozen	Frozen	140-142°F.	142-144°F.	144-146°F.
1	193	196	221	210	223
2	179	194	185	212	220
3	180	174	208	199	207
4	185	214	196	.....	203
Average	184.3	194.5	202.5	207.0	213.3

[1] In 1/10 mm. units with 100 gm. stress applied for 15 seconds.

onnaise, followed in order by the unpasteurized frozen sample and the pasteurized frozen samples. All of the whole egg samples produced mayonnaise which was of satisfactory consistency.

The mayonnaises made with frozen yolk (Table 3) were much stiffer than those made with frozen whole egg (Table 2). The penetrometer weight of 100 gm. used for testing whole egg mayonnaise had to be increased to 150 gm. when the mayonnaise was made with unthinned yolk.

In whole egg mayonnaises, it was noted that the use of the frozen product tended to decrease mayonnaise viscosity (Table 2) while in yolk mayonnaise the opposite was true (Table 3 and Fig. 1). An increase in the pasteurization temperature of yolk resulted in a slight decrease in

TABLE 3

Stiffness of Unthinned Yolk Mayonnaise (Penetrometer Readings)[1]

Replications	Unpasteurized samples		Pasteurized frozen samples	
	Unfrozen	Frozen	140-142°F.	144-146°F.
1	285	133	111	124
2	284	121	119	135
3	247	141	132	130
4	247	129	133	132
5	241	136	135	136
6	245	137	130	138
7	248	141	136	132
8	234	139	135	154
9	220	137	156	160
10	209	143	156	157
Average	246	136	134	140

[1] In 1/10 mm. units with 150 gm. stress applied for 15 seconds.

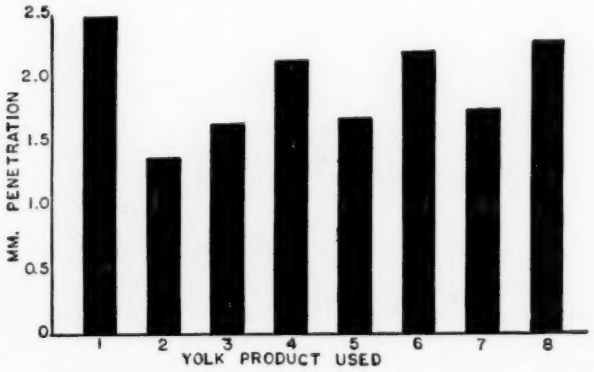

FIG. 1. Stiffness of mayonnaise made with various yolk products. 1. Unfrozen; 2. Frozen; 3. Frozen and diluted 15 per cent; 4. Frozen and diluted 37 per cent; 5. Pasteurized 140 to 142°F., frozen, diluted 15 per cent; 6. Pasteurized 140 to 142°F., frozen, diluted 37 per cent; 7. Pasteurized 144 to 146°F., frozen, diluted 15 per cent; 8. Pasteurized 144 to 146°F., frozen, diluted 37 per cent.

the stiffness of the mayonnaise made with it. Stability of the mayonnaise made with whole egg, after a four-week holding period, is shown in Table 5. Differences in replications were greater than differences between egg treatments. The average of the four replications indicate that the stability of the mayonnaise made with the pasteurized frozen egg was as good as when made with unpasteurized frozen egg.

Mayonnaise made with yolk and held for two weeks was more stable than when prepared with whole egg and held four weeks. Centrifuging for as long as one hour of the unthinned yolk samples failed to separate more than 0.2 to 0.3 ml. of oil. This was true in the case of all the mayon-

TABLE 4

Stiffness of Thinned Yolk Mayonnaise
(Penetrometer Readings)[1]

Replication	Unpasteurized samples			Pasteurized frozen samples			
	Unfrozen	Frozen		140-142°F.		144-146°F.	
	40 gm. yolk	34 gm. yolk	25.5 gm. yolk	34 gm. yolk	25.5 gm. yolk	34 gm. yolk	25.5 gm. yolk
1	188	168	230	167	217	163	191
2	208	164	215	145	198	161	227
3	203	145	188	175	194	165	200
4	198	160	230	174	237	173	245
5	206	161	183	176	231	173	237
6	190	155	184	165	226	182	232
7	196	167	227	170	211	194	237
8	198	172	227	166	229	180	241
9	197	173	242	168	228	176	240
Average	198	163	214	167	219	174	228

[1] In 1/10 mm. units with 100 gm. stress applied for 15 seconds.

TABLE 5

Comparative Stability of Whole Egg Mayonnaise Emulsions
(In Milliliters of Oil Separation)

Replication	Unpasteurized samples		Pasteurized frozen samples		
	Unfrozen	Frozen	140-142°F.	142-144°F.	144-146°F.
1 a	2.50	0.25	0.50	1.50	0.30
b	2.50	0.15	0.70	1.90	0.50
2 a	0.40	0.30	0.20	0.50	0.30
b	0.50	0.40	0.20	0.40	0.30
3 a	0.70	0.60	0.80	0.20	0.60
b	0.50	0.80	0.50	0.50	0.50
4 a	0.90	1.20	0.50	.....	0.40
b	0.70	1.80	0.70	.....	0.20
Average	1.06	0.68	0.51	0.83	0.39

naise samples whether made with unfrozen egg yolk, frozen yolk, or pasteurized frozen yolk.

Results of tests on stability of thinned yolk mayonnaises were similar to those of the unthinned. There was very little variation in 18 trials within each treatment. The average figures were as follows:

	ml. oil
Shell egg yolk (40 gm. yolk)	0.22
Frozen unpasteurized yolk (34 gm. yolk)	0.28
Frozen unpasteurized yolk (25.5 gm. yolk)	0.46
Frozen pasteurized at 140°F. (34 gm. yolk)	0.25
Frozen pasteurized at 140°F. (25.5 gm. yolk)	0.33
Frozen pasteurized at 146°F. (34 gm. yolk)	0.28
Frozen pasteurized at 146°F. (25.5 gm. yolk)	0.39

Although differences were not great, there was a tendency for greater stability with increased yolk content (Fig. 2). It appeared that the amount of frozen yolk used in mayonnaise under the conditions of this

test could be reduced from 13.5 to 8.6 per cent with no marked deleterious effect on the stability or viscosity.

The thinned yolk mayonnaises as well as the unthinned tended to be more stable than those made with whole egg. Snell, Olsen, and Kremmers (1935) have shown that the emulsifying action of egg resides in the unstable lecitho-protein fraction. Since there is greater concentration of this fraction in yolk than in whole egg, one would expect a more stable emulsion in yolk mayonnaise even after thinning the yolk with 20 to 30 per cent water, than in whole egg mayonnaise.

The mayonnaises made with yolk were judged for quality by home economists. They were ranked for stiffness and flavor. In 10 rankings of

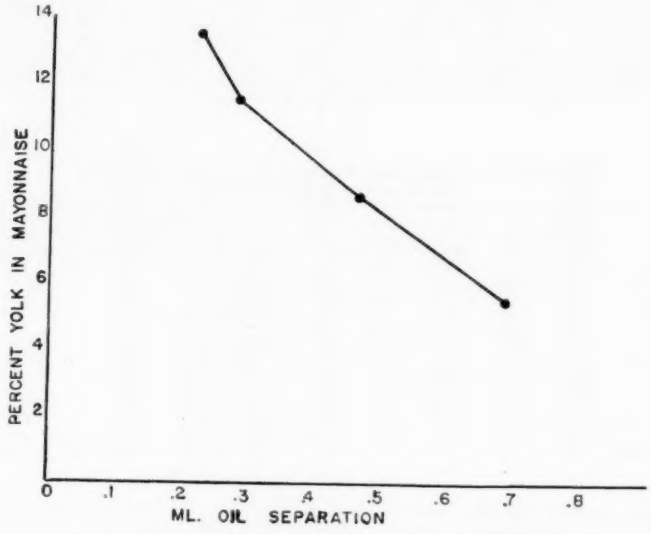

FIG. 2. Influence of yolk content on stability of mayonnaise.

the unthinned yolk products, five of the six judges consistently preferred the stiffness of the unfrozen yolk mayonnaise saying that those made with the frozen yolk were too stiff. One judge preferred the stiff ones. Preferences for flavor varied widely and were not consistent. It was concluded that no real flavor difference existed.

All six judges preferred the appearance of the unfrozen yolk mayonnaise to that made with diluted frozen yolk although penetrometer readings indicated little difference. They may have been influenced by the fact that the thinned frozen yolk samples contained tiny air bubbles and therefore the fresh yolk samples seemed to be smoother. Five of the seven judges preferred the flavor of the pasteurized frozen samples while two felt the unfrozen yolk sample tasted slightly better. No real flavor

differences existed. A number of unofficial judges ranked all samples superior in quality and flavor.

SUMMARY

1. Pasteurization of whole egg and yolk for four minutes at temperatures ranging from 140 to 146°F.(60.0 to 63.3°C.) before freezing did not impair their value for making mayonnaise under the conditions tested when judged by consistency, stability, and palatability of the stored product.

2. Mayonnaise made with 13.5 per cent defrosted plain yolk was too stiff to be readily acceptable by a panel of judges.

3. As the per cent of defrosted yolk in mayonnaise was decreased from 13.5 per cent to 5.5 per cent, the stiffness and stability of the mayonnaise declined.

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THE HEATING OF SUCROSE SOLUTIONS. I. THE RELATIONSHIP OF 5-(HYDROXYMETHYL)-FURFURAL TO COLOR FORMATION[1]

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The heating of sucrose solutions to temperatures above 150°C. produces many changes, including inversion of the sucrose and a yellow or brown discoloration, Ambler and Byall (1935). It is well known that the inversion products, glucose and fructose, undergo further degradation to produce the discolorations in these solutions. One of the most readily identifiable products obtained, 5-(hydroxymethyl)-furfural (HMF), has been found to function under some conditions as an intermediate in the "browning" reaction, Singh, Dean, and Cantor (1948) and Haas, Stadtman, Stadtman, and Mackinney (1948). The fact that HMF and color are formed simultaneously when sucrose solutions are heated, indicates that HMF may be an important contributor to the color formation.

The purpose of this investigation was to ascertain the importance of HMF as a color intermediate in the heating of sucrose solutions to temperatures above 100°C. Since it was desired to study the color formation in its early stages most of the solutions were heated to 165°C. or less.

EXPERIMENTAL

Materials. The sucrose, glucose, and fructose were the purest grades available from Merck and Company.

The 5-(hydroxymethyl)-furfural was prepared from sucrose by the procedure of Haworth and Jones (1944). It was twice distilled under high vacuum (molecular absorption coefficient at 285 mμ was 16,700).

The charcoal, Norit A (Pfahnstiel), was mixed with Cellite 545 (Johns-Manville) (1:2 by weight) and washed successively with 1 *N* hydrochloric acid, 1 *N* sodium hydroxide, 1 *N* hydrochloric acid, and distilled water until free of chloride.

The heated sucrose solutions were prepared as follows: 100 gm. of sucrose was weighed into a 300 ml. round-bottom Pyrex flask and 50 ml. of water was added. A thermometer was inserted into the flask and the solution was heated with a Glas-Col heating mantle. The Powerstat that was used to control the current to the heating mantle was maintained at a setting of 80. When the desired temperature was reached, the solution was quickly poured into 200 ml. of water in a 400 ml. beaker. The flask was cooled and rinsed. The solution and rinsings were made up to 500 ml. The approximate time required for heating the solutions to the different temperatures is given in Table 1.

Methods. The degree of sucrose inversion by heating was determined by hydrolyzing the sucrose with hydrochloric acid, A.O.A.C. (1945), and determining the sugars by the copper reduction method of Phillips and Smith (1943). The acid-hydrolyzed samples were first neutralized with sodium hydroxide.

The color values were determined as follows: Readings were taken in 19 mm. test tubes with a Junior Coleman Spectrophotometer at 440 mμ in the optical density range of 0.0-0.150. As the readings in this range were linear, it was possible to calculate the

[1] Acknowledgment is made to E. J. Brach and Sons, Chicago, Illinois, for financial assistance in this work.

TABLE 1

Approximate Times of Heating Used at Different Temperatures

Heating temperature, °C.	Total time, seconds	Total time after reaching boiling point, seconds	Time for each step, seconds
(Boiling)	570	0	570
130	1635	1065	1065
145	1918	1348	283
155	2051	1481	133
165	2151	1581	100
175	2230	1660	79
185	2286	1716	56

color as total color based on the density readings. For example, if the original density reading at a dilution of 1:10 was 0.122, the total color would be: $500 \times 10 \times 0.122 = 610$.

The heated sucrose solutions were chromatographed as follows: Four to six gm. of the charcoal-Cellite mixture was placed in a 19 × 200 mm. chromatographic column. An amount of sucrose solution containing a total color of 10 per gm. of adsorbent was diluted 1:4 with distilled water, and passed through the adsorbent. The column was washed with 2,000 ml. of water and eluted with 400 ml. of methanol.

The Beckman spectrophotometer (Model DU) was used for the ultraviolet absorption studies. One cm. fused silica cells were used. Measurements were made in the region 210-320 mμ at slit widths of 1.8 mm. at 210 mμ to 0.36-0.38 mm. at 320 mμ. The slit width at 285 mμ was 0.40-0.42 mm. The solvent was water and the pH was between 4 and 5. The apparent HMF of the solutions was calculated from the formula $\epsilon = 1/c \log(100/T)$, where ϵ is the molecular absorption coefficient, c the moles per liter, and T the per cent transmission. The T used in this paper was that at 285 mμ.

RESULTS AND DISCUSSION

There is no completely satisfactory method of following the formation of HMF when sugar solutions are heated. The fact that HMF has a specific absorption curve in the ultraviolet region and has a very high absorption coefficient has been used by Singh, Dean, and Cantor (1948) to study its formation from glucose. This appears to be satisfactory where the content of HMF is sufficient to give an absorption curve similar to that of HMF. In many of the solutions with which we were dealing this was not the case and an additional method was necessary to give a more reliable estimation of the HMF present.

It was found that HMF could be adsorbed on charcoal from water solutions and subsequently eluted with methanol with good recoveries (Table 2). When heated sucrose solutions were chromatographed in the same manner, all of the color and most of the substances responsible for the high absorption in the 230 mμ region were removed from the methanol fraction (Figs. 1 and 2). The ultraviolet absorption curves of the methanol

TABLE 2

Recovery of HMF from Charcoal

Amount adsorbed	Amount eluted	Recovery
105.8 mg.	99.14 mg.	93.7 per cent
9.87 mg.	9.24 mg.	93.6 per cent

eluate showed that this method did not provide complete purification of the HMF (Fig. 3), but it did yield results that were much nearer to the true values of the HMF present (Table 3).

Ultraviolet light absorption observations during the course of the heating of sucrose solutions showed a light absorption pattern (Fig. 4) similar

TABLE 3

Charcoal Fractionation of Heated Sucrose Solutions

Total color	Calculated HMF before adsorption,	Calculated HMF methanol fraction,	Calculated HMF methanol fraction,
	mg.	mg.	pct.
7	1.2	0.12	10.0
17	3.1	0.74	23.6
31	15.4	5.6	36.4
58	23.1	11.7	50.6
124	76.3	47.7	62.5
384	255.5	185.5	72.6

to that found by Wolfrom, Schuetz, and Cavalieri (1948) and Singh, Dean, and Cantor (1948) for the heating of glucose solutions. First there was increased absorption in the 230 mμ region, followed by a gradual increase in the 285 mμ region until an absorption spectrum similar to that of HMF was obtained. This indicated an increased accumulation of HMF in the solutions as the temperature to which the solutions were heated was increased. This accumulation was also indicated when the absorption at 285 mμ was calculated as HMF (Table 4). This increase in HMF followed closely the hydrolysis of the sucrose as is shown in Table 4. This relation-

TABLE 4

The Effect of Temperature on Heating Sucrose

Heating temperature, °C.	Color	Hydrolysis,	Calculated HMF,	Time in seconds
		pct.	mg.	
(Boiling)	0	0	0	570
145	2.5	2.0	....	1918
155	13	5.8	0.4	2051
165	31	13.7	5.6	2151
175	89	29.4	31.5	2230
185	190	49.1	78.9	2286

ship is what would be expected since Singh, Dean, and Cantor (1948) have indicated that the formation of HMF from glucose is a first order reaction. Fructose is probably much more important in the reaction here than glucose, but one would expect that the formation of HMF from fructose would, also, be a first order reaction.

The color formation in the heated sucrose solutions was closely related to the hydrolysis of the sucrose and the formation of HMF (Table 4). This relationship might indicate that HMF was directly responsible for the color formation. Teunissen (1930) has shown that in acid solutions at 100°C. HMF is hydrolyzed by a first order reaction to formic and levulinic

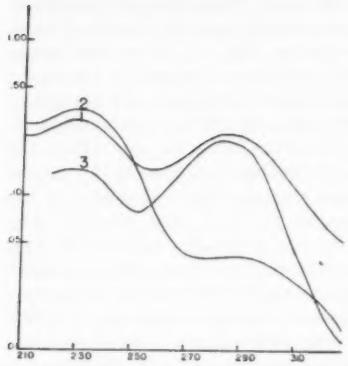

FIG. 1. Ultraviolet absorption spectra of the charcoal fractionation of a sucrose solution heated to 165°C. Total color=42. 1. Original solution. 2. Filtrate. 3. Methanol eluate.

Vertical axis—optical density.

Horizontal axis—wave length—millimicrons.

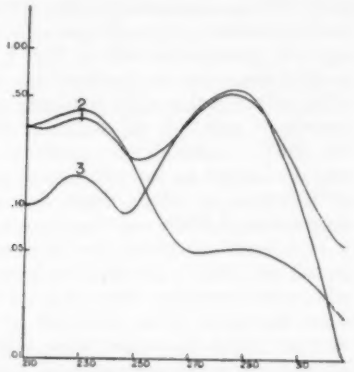

FIG. 2. Ultraviolet absorption spectra of the charcoal fractionation of a sucrose solution heated to 165°C. Total color=41, with 23.2 mg. HMF added before heating. 1. Original solution. 2. Filtrate. 3. Methanol eluate.

Vertical axis—optical density.

Horizontal axis—wave length—millimicrons.

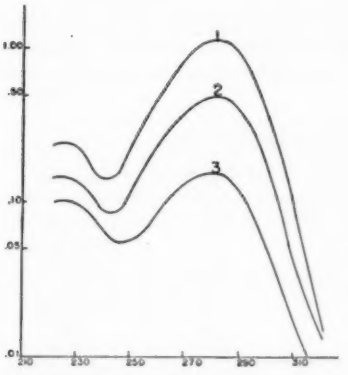

FIG. 3. Ultraviolet absorption spectra of HMF in methanol from charcoal fractionation of heated sucrose solutions. 1. HMF. 2. Sucrose, 23.2 mg. HMF, 165°. 3. Sucrose, 165°.

Vertical axis—optical density.

Horizontal axis—wave length—millimicrons.

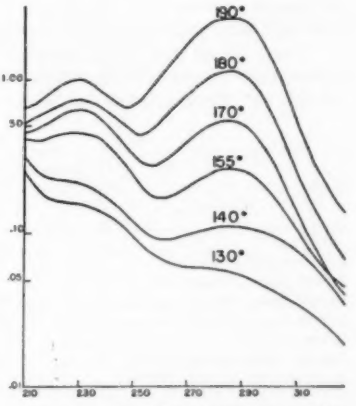

FIG. 4. Ultraviolet absorption spectra of heated sucrose solutions.

Vertical axis—optical density.

Horizontal axis—wave length—millimicrons.

acids with an accompanying formation of color. The degree of hydrolysis was dependent on the acid used, its concentration, and the length of heating. He found that 0.001 *N* H_2SO_4 heated at 100° C. for several hours would produce a very small amount of hydrolysis and color in a 1 per cent HMF solution, that a 0.5 *N* formic acid solution produced only a trace of hydrolysis, and that the heating of HMF alone at 100°C. had no effect on the HMF. Although no quantitative estimation of color was made, the amount formed in the solutions seemed to follow very closely the degree of hydrolysis of HMF. Singh, Dean, and Cantor (1948) showed that the destruction of HMF and the color formed in a 0.35 per cent solution, when it was heated at 145°C. for 30 minutes, was dependent on the pH (adjusted with HCl). At pH 2.88 there was 12 per cent destruction of HMF with color formation while at pH 4.0 there was no destruction and only a small amount of color produced. It appears that the hydrolysis of HMF and the color formation from HMF are very closely related and are affected by the same conditions. Since the conditions [2] prevailing in this investigation were not conducive to the hydrolysis of HMF, it might be considered doubtful that HMF contributed much to the color formation in the heated sucrose solutions.

Since there was no way to test the effect of the heating conditions used here on HMF alone, it was necessary to determine the fate of HMF added to the sucrose solutions before they were heated. If HMF alone or its degradation products were responsible for the color formation, the addition of HMF should result in more color formed with an appreciable loss in the added HMF. Also, the ratio of the color to the calculated HMF in the heated sucrose solution should be approximately the same for both the solutions with and without added HMF.

It was found that the addition of 3-30 mg. of HMF to sucrose solutions before they were heated to 165°C. had little effect on the color formation (Table 5). Furthermore, the ratio of the color formed to the calculated

TABLE 5

Color: HMF Ratios in Solutions Heated to 165°C.

Treatment	Total color	Calculated HMF,	Color/HMF
		mg.	
Invert sugar, 15 per cent	108	52.2	2.07
Invert sugar, 25 per cent	140	67.2	2.08
Invert sugar, 50 per cent	222	103.3	2.15
Invert sugar, 100 per cent	535	251.0	2.13
Sucrose	31	5.6	5.54
Sucrose, plus 13.8 mg. HMF	32	16.6	1.93
Sucrose, plus 550 mg. HMF	83	470.0	0.18

HMF was much lower in all the samples where HMF had been added. There was approximately a 20 per cent loss of the HMF added to the sucrose solutions during the course of the heating. This loss is not as surprising as the lack of its effect on the color formation. Only when samples

[2] No mineral acids present, the pH not less than 3.5, HMF present for only a very short time.

containing much larger amounts of HMF (550 mg.) were heated to 165°C., was there an appreciable increase in color. In these samples there was a loss of about 16 per cent (86 mg.) of the added HMF with an increase in color of 52. Thus, for every mg. of HMF destroyed there was an increase of color of 0.6. If this same ratio holds for the smaller amounts of HMF added, a maximum increase in color of 2 would have been obtained.

When invert sugar was substituted for some of the sucrose in the heating experiments, there was an increase in color and calculated HMF in proportion to the amount of invert sugar added. Since it has already been determined that only 16 to 20 per cent of any HMF present is destroyed under these conditions of heating, it can be calculated that the maximum amount of HMF that could have been present in the solution containing 100 per cent invert sugar was 300 mg. This solution contained 6-7 times as much color as the sample to which 550 mg. of HMF was added, indicating that the color formation occurred through some other course than HMF alone.

The above results show that when sucrose solutions are heated to 165°C., a very minor portion of the color produced is due to HMF alone. This does not necessarily eliminate HMF from further considerations because HMF may combine with some other sugar degradation product(s) to produce color. However, it does indicate that there are other compounds that are at least as important as HMF in color formation in sucrose solutions heated to 165°C.

SUMMARY

1. The estimation of the HMF in heated sucrose solutions was improved when the solutions were chromatographed on charcoal.

2. The production and accumulation of HMF increased in heated sucrose solutions as the temperature to which the solutions were heated was increased.

3. The formation of color in heated sucrose solutions was simultaneous with hydrolysis of the sucrose and HMF formation.

4. The addition of HMF in quantities equivalent to those produced in heated sucrose solutions did not affect the color formation. More color was produced by the substitution of invert sugar for sucrose than by the addition of HMF.

5. It was concluded that other compounds present were at least as important as HMF in color formation.

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CONSUMER PREFERENCE FOR BREAD CONTAINING DIFFERENT LEVELS OF NON-FAT DRY MILK SOLIDS[1]

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Comparing preferences for bread with no milk and with 6 per cent non-fat dry milk solids added (based on flour weight as 100 per cent), Bailey (1939) found that approximately 900 young men between 16 and 35 years of age averaged 3.29 per cent greater consumption of the bread with the non-fat dry milk solids. In a further study of 220 boys between 9 and 24 years of age, the preference for bread with dry milk solids was 5 per cent greater. Cafeteria customers, given free choice, selected milk bread in the proportion of 6 to 4.

To determine the preference for bread with varying levels of non-fat milk solids and to get some indication of the optimum level for maximum bread consumption, the studies reported in this paper were undertaken at a boys' school in California.

The conditions at this school satisfied several of the criteria necessary for reliable observations. The dietary regime of the boys was closely controlled and the regular diet was adequate, varietally and nutritionally. Bread of uniform and standard high quality was baked regularly in the school bakeshop and was offered freely at all meals. A reasonable number of subjects, about 320 boys, aged 8 to 16 years, was used for the study. Moreover, the facilities were available for a considerable period of time so that possible seasonal differences could be balanced out.

EXPERIMENTAL PROCEDURE

The experiment was set up to determine the per capita consumption of bread per day over an eight-week period when each of the following levels of non-fat dry milk solids was incorporated in the bread: 0, 6, 10, and 14 per cent, based on the flour as 100 per cent.

An eight-week period, it was believed, would reflect the continuing preference for the differnet levels of milk solids. Also, the different levels of milk solids were considered sufficiently far apart to reflect any significant differences.

In conducting the experiment, the regular bread containing no non-fat dry milk solids was fed and data were collected for an eight-week period. The 6 per cent milk bread was given for the next eight-week period, and was followed by the 10 per cent and 14 per cent milk breads in that order. Then, to minimize any possible effects of seasonal or cumulative influence, each of the breads was fed for an additional eight-week period in a different order: 6 per cent, 14 per cent, 10 per cent, and 0 per cent. Consumption records were thus obtained for a total of 16 weeks for each bread.

Made by the straight dough process, the bread included the usual dough ingredients, the flour being enriched to the levels recommended by the Food and Nutrition Board of the National Research Council. Except for the 14 per cent bread, the same formula was used for all breads having different amounts of non-fat dry milk solids. A sufficient quantity of water, of course, had to be used to give the proper consistency. The

[1] This work was supported in part by funds from the California Dairy Industry Advisory Board.

TABLE 1

Typical Weekly Menu

	Breakfast	Lunch	Dinner
Monday	Bread Hot Cakes Syrup Prunes Margarine Cocoa	Bread Macaroni Beets Salad Rice Pudding Milk	Bread Stew Potatoes Cabbage Pears Cake Milk Margarine
Tuesday	Bread Fruit Cup Cake Rice Margarine Cocoa Sugar Milk	Bread Beans Cauliflower Carrots Cracklings Pudding Milk	Bread Onions Asparagus Broccoli Carrot Sticks Margarine Cake Milk Goulash
Wednesday	Bread Fruit Bacon Eggs Cup Cake Margarine Cocoa	Bread Weiners Beans Cauliflower Carrots Onions Pudding Milk	Bread Liver Onions Potatoes Salad Margarine Cookies Oranges Milk
Thursday	Bread French Toast Jam Margarine Fruit Cocoa	Bread Liver Macaroni Potatoes String Beans French Toast Salad Pudding Milk	Bread Beef Potato Slaw Margarine Oranges Cake Milk
Friday	Bread Fruit Cereal Sugar Milk Cup Cake Margarine Cocoa	Bread Rice Cauliflower Carrots Cracklings Eclairs Milk	Bread Fish Potatoes Tomatoes Cottage Cheese Margarine Pudding Oranges Milk
Saturday	Bread Eggs Bacon Prunes Cup Cake Margarine Cocoa	Bread Lima Beans Broccoli Beets Cup Cake Milk	Bread Oranges Peas Beans Chilibeans Potatoes Salad Margarine Milk Cookies
Sunday	Bread Cereal Milk Peaches Cup Cake Margarine Cocoa	Bread Lamb Chops Gravy Roast Lamb Peas Potatoes Avocado Margarine Apple cobbler Milk	Bread Soup Bologna Crackers Cheese Margarine Pickles Oranges Cake Milk

14 per cent bread darkened on the crust. To overcome this excessively dark exterior, the sugar was slightly reduced. The loaves were rectangular and of standard 1½-pound size. The size and appearance of the loaves with the different levels of non-fat dry milk solids were not noticeably different. All bread was baked to a deep brown color. Bakings were made regularly 3 to 4 times weekly.

TABLE 2

Daily Bread and Total Food Consumption

Per cent of non-fat dry milk solids in the bread		Average daily bread consumption, gm.	Average daily food consumption, gm.
First trial	0	203	2,320
	6	210	2,425
	10	219	2,305
	14	232	2,220
Second trial	0	240	2,400
	6	252	2,220
	10	256	2,210
	14	265	2,225

All food consumed, as well as all bread eaten, was weighed accurately. Attendants were careful to gather all scraps, so that their weight could be subtracted from the weights served. To check on the nutritional adequacy of the diets, aliquot food samples of each meal were taken and composited for chemical analysis. Menus for each meal were prepared in advance and were preserved as part of the record of the experiment. A typical menu is shown in Table 1.

Since a work detail of 3 to 4 boys was used regularly in the school bakery, it was not possible to prevent some knowledge of the inclusion of non-fat dry milk solids. They were not aware, however, of the regularity of any changes made in the bread, nor was there ever any discussion among the boys generally to indicate that they realized that the bread was altered. It is believed also that the length of each feeding period, eight weeks, eliminated any minor psychological influences.

TABLE 3

Daily Amounts of Food Nutrients

Per cent of non-fat dry milk solids in the bread		Solids in food		Protein in food		Calories[1]	Ca, gm.	P, gm.
		pct.	gm.	pct.	gm.			
First trial	0	31.2	723	4.84	112	3,243	2.38	2.69
	6	30.2	734	5.06	123	3,299	3.00	2.50
	10	30.7	709	5.04	116	3,183	2.56	2.75
	14	31.7	702	5.38	120	3,145	2.92	2.60
Second trial	0	30.0	721	5.27	126	3,241	2.40	2.40
	6	32.7	725	5.30	118	3,197	2.50	2.50
	10	32.3	713	5.40	119	3,200	2.54	2.60
	14	32.1	715	5.46	122	3,281	2.39	2.37

[1] Calculated.

The composite food samples were analyzed for moisture, protein, calcium, phosphorus, ash, and occasionally for thiamine and riboflavin.

EXPERIMENTAL RESULTS

The consumption of bread and food during the eighth experimental periods of 8 weeks each is shown in Table 2.

It will be noted that in each trial the consumption of bread increased with the increase of milk solids, while the total food consumption was

fairly uniform. During the second trial the boys ate consistently more bread at all levels of milk solids than during the first trial. The authors have no satisfactory explanation for this fact.

The bread consumption on a percentage basis, with the 0 per cent milk solids bread at 100 per cent, is shown in Figure 1.

The composition of the food is shown in Table 3.

The food nutrients in the diet were adequate according to accepted standards for all constituents analyzed. The Food and Nutrition Board of the National Research Council recommends a daily caloric intake of 2,500 to 3,200 calories for boys in this age group. The average intake was at the higher figure throughout the experiment. The recommended daily protein intake is 70 to 85 gm.; the diet supplied was well in excess of 100 gm. Calcium was also liberal, exceeding 2 gm. daily, with a recommended

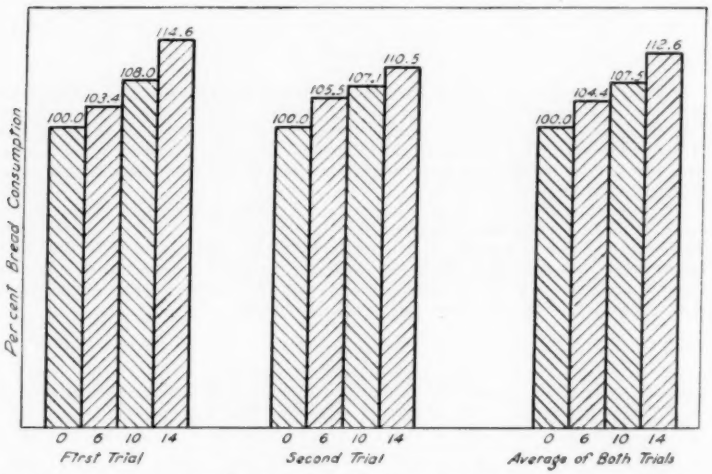

FIG. 1. Per cent of non-fat dry milk solids in the bread on average bread consumption.

allowance of 1.4 gm. The riboflavin and thiamine values obtained periodically during the experiment ranged from 2.0 to 2.5 mg. of riboflavin daily and from 1.5 to 2.0 mg. of thiamine, whereas the recommended allowances are 2.0 mg. and 1.5 mg., respectively. It is apparent, therefore, that the diets were liberal both in variety and in total composition.

DISCUSSION OF RESULTS

The data presented here substantiate the results of Bailey (1939) in indicating a distinct preference for bread containing non-fat dry milk solids. They show, in addition, that as the amount of milk solids is increased up to 14 per cent, this preference is accentuated. Whether or not this is the optimum amount of milk solids with respect to maximum bread consumption could not be determined from these data. In view both of

the consistent results from the trials and the safeguards used to assure reliability of the observations, these preferences are significant, particularly where bread forms a substantial part of the diet.

This experiment extended over an eighteen-month period, covering the two trials with a short interval between them. The continuity of the data had only two interruptions, both resulting from abnormal conditions. One occurred in the first trial—during a 10-day period over the Christmas holidays, when 14 per cent milk bread was being fed. For various reasons, the bread during this 10-day period was not at its usual freshness. The consumption data for this short time were disregarded, and the 14 per cent time was extended to make the full eight weeks on bread of normal characteristics. The other interruption occurred during the second trial on 10 per cent milk-solids bread. The school baker was absent for three weeks, and no competent substitute was available. Commercial bread was purchased during this time. The data were not used, inasmuch as the character of the bread differed somewhat from that of the school manufacture.

SUMMARY AND CONCLUSIONS

1. Bread containing 0, 6, 10, and 14 per cent non-fat dry milk solids, based on the flour, was fed to approximately 320 boys aged 8 to 16 years, in two trials of eight weeks for each kind of bread.

2. In the first trial, using 100 per cent for the consumption of bread containing no non-fat dry milk solids, the consumption was 103.4 per cent for the 6 per cent, 108.0 per cent for the 10 per cent, and 114.6 per cent for the 14 per cent milk solids bread.

3. In the second trial the consumption was 105.5 for the 6 per cent bread, 107.1 for the 10 per cent bread, and 110.5 for the 14 per cent, expressed on the same basis.

4. The average of the two trials showed a consumption of 104.4, 107.5, and 112.6 per cent for the 6, 10, and 14 per cent milk-solids breads, respectively, using again 100 per cent for the consumption of the control bread.

5. Examination of the menus and analyses of the food showed that during these experiments the diets were liberal in variety and nutritionally adequate.

ACKNOWLEDGMENTS

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THE OCCURRENCE OF SUBSTANCES WHICH INTERFERE WITH THE DETERMINATION OF ASCORBIC ACID IN ANTHOCYANIN-CONTAINING PLANT PRODUCTS

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The determination of ascorbic acid in anthocyanin-containing plant products using 2,6-dichlorobenzenone indophenol presents special problems. Extracts of such products are approximately the same color as that of the dye in the acidic solutions customarily used to extract ascorbic acid. The indophenol-xylene extraction method, originally described by Bukatsch (1939) obviates this difficulty since it makes it possible to separate the dye from the anthocyanin. The excess dye is extracted with xylene and the anthocyanins are left in the aqueous phase. Pepkowitz, Larson, Gardner, and Owens (1944) used this method to measure the ascorbic acid content of a number of vegetables, including red garden beets. Miller (1947) similarly used this method with a number of fresh and canned foods. She also included the formaldehyde fixation procedure as described by Robinson and Stotz (1945) to detect the presence of interfering substances, which she grouped under the heading "reductones." No interference was found with any of the fresh products, although beets were included. She found interfering substances to be present in a number of canned and processed products, among which were anthocyanin-containing products such as cranberry sauce and canned beets, but a number of other products which contain no anthocyanins also showed interference. Other workers have reported ascorbic acid values for anthocyanin-containing products. For example, Griggs and Rollins (1948) analyzed cultivated blueberries using the procedure of Loeffler and Ponting (1942). This observation is pertinent to the results presented below, but it probably is not necessary to cite all of the research dealing with the ascorbic acid content of anthocyanin-containing products. Many plants and plant products contain small amounts of these pigments.

The results reported below were presented in brief form earlier by Somers, Kelly, Thacker, and Redder (1949) where it was pointed out that anthocyanin-containing plant products contain substances which react with the indophenol dye. These substances can lead to erroneous values for the ascorbic acid content of these products unless suitable precautions are taken. In the present paper data are presented to support such a conclusion.

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METHODS AND MATERIALS

Ascorbic acid was determined by the indophenol-xylene extraction method of Nelson and Somers (1945), modified, where appropriate, to include formaldehyde fixation as described by Snow and Zilva (1943). In this modified procedure, an aliquot of the extract is diluted to 5 ml. with the extractant, e.g. 3 per cent metaphosphoric acid; then 5 ml. of acetate buffer (as described by Nelson and Somers, diluted 2.5 times) and 0.5 ml. of formalin are added. The pH of the buffered aliquot is about 3.9. After 10 minutes at room temperature the analysis is completed in the usual way. This method is very similar to that described by Robinson and Stotz (1945), except that these workers used a somewhat greater concentration of formaldehyde. It was found that the smaller amount used here was adequate to render essentially all of the ascorbic acid nonreactive. It was found necessary to control the reaction time, i.e. the length of time the dye was allowed to react with the buffered aliquot of the extract, both in the presence and absence of formaldehyde.

All chemicals used were reagent grade, except for some lots of metaphosphoric acid which were technical grade. This grade of metaphosphoric acid appeared to be as satisfactory as the reagent grade. Care must be taken to avoid some of the metaphosphoric acid now being sold, since it contains only 35 per cent of the free acid. The 2,6-dichlorobenzenone indophenol was obtained from Eastman Kodak. Formaldehyde was obtained in the usual aqueous solutions, formalin U.S.P., containing about 40 per cent formaldehyde.

The plant materials were obtained from various sources. All of the beets were red garden beets of several varieties; some were grown in local garden plots, some were grown in sand culture, and some were obtained from a local market. The sources or variety of the beets seemed to be of little consequence. The chard varieties (*Beta vulgaris* L.) were grown in sand culture in the green house. The cabbage varieties (*Brassica oleracea* L.) and the cultivated blueberries (*Vaccinium corymbosum* L.) were purchased from a local market. The other fruits which were used were picked from plants growing nearby by laboratory personnel, and were as follows: elderberries (*Sambucus canadensis* L.); red raspberries (*Rubus idaeus* var. *strigosus* Michx.); black raspberries (*Rubus occidentalis* L.); blackberries (*Rubus allegheniensis* Porter); black currants (*Ribes sp.*); wild blueberries (*Vaccinium corymbosum* L.); and chokecherries (*Prunus virginiana* L.).

The following anthocyanin preparations were used in the course of this investigation:

1. A crude betanin prepared from beet hypocotyls by the method of Pucher, Curtis, and Vickery (1938).

2. Highly purified betanin obtained from Dr. Pucher. This had been isolated a number of years earlier from red garden beets (Pucher, Curtis, and Vickery, 1938). When originally prepared, this material was reported to be readily soluble in water, but when it was used in the analyses reported below it apparently had decomposed partially since it contained a fraction only sparingly soluble in 3 per cent metaphosphoric acid or water.

3. A betanin concentrate prepared from red garden beets in large amounts by the procedure outlined by Somers, *et al.* (1949). This was obtained by freeze-drying the juice pressed from frozen, grated beets. The resulting material was dissolved in water, the solution was adjusted to pH 3.5, and the anthocyanin was precipitated again by adding four volumes of 95 per cent ethanol. The precipitate was washed first with 80 per cent and then with absolute ethanol and finally with acetone, and was dried *in vacuo*. By this procedure the large amounts of betanin required for bioassay purposes could be prepared more readily than by the procedure used for the above preparations, and in addition the use of lithium and lead salts was avoided. It seemed desirable to avoid the use of large amounts of such salts in the preparation of any material to be used for bioassay purposes.

4. A solution of anthocyanin prepared from elderberries. To the juice pressed from ripe berries frozen at −18°C. enough ethanol was added to give a concentration of 90 per cent. After this solution had been filtered, barium acetate (pH 5.0) was added and the resulting precipitate was removed by centrifuging. The anthocyanin was precipitated from the supernatant with lead acetate and was separated by centrifuging. This precipitate was suspended in water and was decomposed with HCl. The lead chlo-

ride was removed by centrifuging. The anthocyanin content of the final solution, as measured in an Evelyn colorimeter with a No. 515 filter, was about 75 per cent that of the original press juice.

The betanin preparation No. 3 was used for bioassay. The bioassay selected was the depletion technique as used by Tressler, Mack, and King (1936). In accordance with this procedure, 30 guinea pigs weighing between 225 and 450 gm. were distributed among the contemplated test groups in individual cages and offered the scorbutogenic basal diet *ad libitum* plus 1 mg. ascorbic acid daily administered orally by syringe. The animals were weighed biweekly and their condition observed at each weighing.

It soon became evident that the basal Sherman and Smith diet supplemented with ascorbic acid was not adequate to maintain the guinea pig. The animals lost weight steadily and several individuals suffered an oral discharge of increasing severity as evidenced by the progressive involvement of larger areas of dampness about the mouth and ventral surface of the neck. Supplementing the basal diet with a complete vitamin mixture did not improve the condition of the pigs. After the death of 9 animals between 14 and 19 days on the diet, the remaining guinea pigs were offered the stock diet (1:1 by weight of whole grain wheat and oats with alfalfa hay *ad lib.*) plus 1 mg. ascorbic acid daily. Within a week the remaining animals commenced to gain weight and the oral discharge disappeared. The reason for our inability to maintain the guinea pig on Sherman and Smith's diet is not apparent. However, the nutritive value of the diet may have been adversely affected by overheating of the skim milk powder. Approximately one-sixth of the skim milk powder was scorched when heated in a large air-blast drying oven.

The bioassay was continued by the use of the stock ration. After 10 days on this diet, an essentially normal growth rate was established and the daily ascorbic acid supplement was discontinued. After 18 days on the stock diet without supplementation, a decided weight loss had been suffered by all of the animals, and test feeding was started. The 21 remaining animals were divided into three groups: 5 animals to receive the stock diet only, 5 animals to receive the stock diet + 0.5 mg. ascorbic acid daily per pig, and 11 animals to receive the stock diet + 250 mg. per animal of powdered anthocyanin isolated from beets which was equivalent to 1 mg. ascorbic acid by chemical assay. The anthocyanin and ascorbic acid were taken up in water and fed by syringe.

In describing the results of the chemical analyses, some arbitrary terms are used. Thus, *total apparent ascorbic acid* refers to the amount of ascorbic acid which is equivalent to the total amount of dye reduced by the buffered aliquot of the extract, whether the dye is reduced by ascorbic acid or by some other substance. *Residual reducing capacity* is the capacity of the buffered aliquot of the extract to reduce the dye following formaldehyde fixation. *"True" ascorbic acid* is the difference between the total apparent ascorbic acid and the residual reducing capacity.

RESULTS AND DISCUSSION

The reaction of metaphosphoric acid extracts of fresh red beet hypocotyls (erroneously called roots) with the indophenol dye was found to be characterized as follows: (1) The total apparent ascorbic acid content of the tissue (a) decreased as the amount of tissue per unit volume of extractant was increased, (b) decreased as the size aliquot used for analysis was increased, and (c) increased as the reaction time was increased, at least up to one minute. (2) An appreciable proportion of the reducing capacity of the extract was not fixed by formaldehyde under the conditions described by Snow and Zilva (1943). (3) The recovery of added ascorbic acid was low. These factors influenced the results significantly, as judged by statistical methods, regardless of whether 3 per cent metaphosphoric acid, 1.25 per cent oxalic acid, a mixture of 2 per cent metaphosphoric and 5 per cent sulfuric acid, or 6 per cent metaphosphoric acid was used as the extractant. The different extractants did not yield identical results for the total apparent ascorbic acid content, but when the

"true" ascorbic acid content was determined using formaldehyde fixation reasonably consistent results were obtained (Table 1).

The high correlation coefficient (Somers, *et al.*, 1949) between the residual reducing capacity following formaldehyde fixation and the relative intensity of the red color of the various extracts led to an investigation of the anthocyanin itself. A solution of the crude betanin preparation (No. 1, above) reacted with the indophenol dye at a slow rate, much as did the extracts from fresh beets, and the reducing capacity of this solution was

TABLE 1

"True" Ascorbic Acid Content of Beet Root. A Comparison of Results Obtained Using Various Extractants, Controlled Reaction Times, and Formaldehyde Fixation

Aliquot size	Reaction time	"True" ascorbic acid content, mg./100 gm. fresh weight		
		Extractant		
		HPO_3, 3%	Oxalic acid, 1.25%	HPO_3, 2%, plus H_2SO_4, 5%
3 ml.	10 sec.	3.23	3.20	3.50
	20 sec.	3.50	3.63	3.33
5 ml.	10 sec.	3.00	3.18	3.08
	20 sec.	3.20	3.40	2.73

affected but little by formaldehyde. Approximately 90 per cent of the pure ascorbic acid added to such a solution could be recovered by analysis. Also, the ascorbic acid was rendered essentially non-reactive (to about 5 per cent of the original value) by formaldehyde in the presence of betanin. The soluble fraction of betanin preparation No. 2 also reacted with the indophenol dye in a manner similar to that observed with the interfering substance in extracts from beets. The results, expressed in terms of apparent ascorbic acid, are summarized in Table 2. It is particularly pertinent

TABLE 2

Results Obtained When Betanin Preparation No. 2 Was Allowed to React With Indophenol Dye With and Without Prior Fixation With Formaldehyde

Reaction time	Total apparent ascorbic acid content, mg./gm.			
	20 seconds		40 seconds	
Aliquot size:	$+CH_2O$	$-CH_2O$	$+CH_2O$	$-CH_2O$
2 ml.	29.0	28.3	35.1	33.2
4 ml.	26.6	23.3	30.8	27.6

that the reactivity of the betanin is not reduced by standing 10 minutes in the presence of formaldehyde and acetate buffer at pH 3.9; rather its reactivity appears to be increased slightly (the average increase was 7.3 per cent).

The betanin concentrate No. 3, prepared primarily for bioassay purposes, had the following properties: it was a deep red color and was readily soluble in water. According to colorimetric determinations, it was about

35 per cent as pure as the soluble fraction of preparation No. 2. The absorption curve of a 3 per cent metaphosphoric acid solution (Beckman spectrophotometer) was similar to that given for pigment C from beet tissue by Aronoff and Aronoff (1948), at least over the range measured (from 3500 to 6100 Å). Maximum absorption occurred at 5300 Å. A 3 per cent metaphosphoric acid extract of fresh beet tissue gave a very similar curve with a maximum absorption at the same wave length. Small additional peaks occurred in the beet extract at about 4100, 4500, and 4850 Å. The soluble fraction of the highly purified betanin from Pucher, when dissolved in 3 per cent metaphosphoric acid, gave a maximum absorption at 5100 Å with a secondary peak at 4150 Å. This latter was attributed to the relatively insoluble fraction which presumably resulted from partial decomposition of the original material.

When an aqueous solution of preparation No. 3 was chromatographed on a mixture of talc and Supercel (equal proportions by volume) and developed with distilled water the principle band which showed up was a bright purple one. This was approximately in the position given by pigments C and D by Aronoff and Aronoff (1948). Below this was a yellow band (cf., pigment A of Aronoff and Aronoff) and above it were three bands, an orange, an orange-purple, and a yellow in that order, with the yellow band uppermost. Chromatographing from a 3 per cent metaphosphoric acid solution also showed that the preparation was a mixture. Similarly, the highly purified betanin obtained from Pucher proved to be a mixture of several pigments when chromatographed under similar conditions. In this case at least three distinct purple bands, in addition to an orange one and a yellow one (uppermost in the column) were observed. In some cases in certain stages of the development of the column there seemed to be other bands present also.

The material isolated for animal assay purposes reacted slowly with the indophenol dye and gave essentially the same reaction characteristics as preparation No. 2. Chemical assay indicated a reducing power equivalent to about 3.0 mg. of ascorbic acid per gram with a 10-second reaction time and 6.0 mg. per gram with a 60-second reaction time. Furthermore, it was established that that component which appeared as a purple band on the chromatograph column reacted in a similar manner with the indophenol dye. The less intense bands were not assayed. The results of a bioassay, summarized in Figure 1, show that this preparation had no capacity to alleviate scorbutic symptoms in guinea pigs. The data presented thus far indicate rather clearly that at least one of the pigments present in beet hypocotyls reacts with the indophenol dye, but that this reaction can be distinguished from that of ascorbic acid by the use of formaldehyde and the failure of the pigment concentrate to alleviate scorbutic symptoms in guinea pigs.

The anthocyanin in beets is a rather unusual one since it has been reported to contain nitrogen (see Pucher, *et al.*, 1938). Hence it seemed worthwhile to see if other anthocyanin-containing materials showed a similar reaction with the indophenol dye. For this purpose it was necessary to use carefully controlled reaction times and formaldehyde fixation.

A comparison of results obtained with beet hypocotyls and beet tops is shown in Table 3. These data were obtained by using a 20-second reaction time. A number of other anthocyanin-containing products were similarly assayed (see Table 4). In the case of chard and cabbage, it was

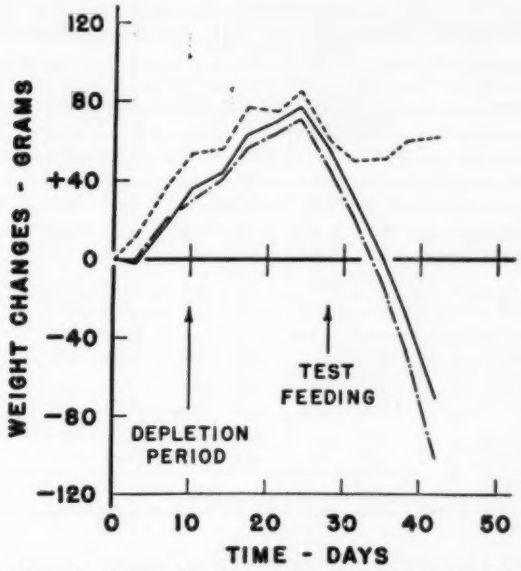

FIG. 1. Guinea pig bioassay for antiscorbutic properties of an anthocyanin preparation obtained from red beets. Weight changes (5 animals per treatment): 0.5 mg. ascorbic acid per day – – – – – –; no supplement – —— – —— – ——; 1 mg. of apparent ascorbic acid as anthocyanin preparation ————————.

possible to compare anthocyanin-free and anthocyanin-containing varieties of the same species. Ruby King chard and red cabbage contain anthocyanin whereas the other varieties tested do not. All of the materials listed in Table 4 were extracted with 3 per cent metaphosphoric acid, and 2 and 5 ml. aliquots were analyzed from each, using 10-, 20-, and 40-second reaction times both in the presence and absence of formaldehyde. In addition, the recovery of pure ascorbic acid which was added to 2 ml. aliquots was measured. With all the products the true ascorbic acid content, i.e. corrected for reducing substances not fixed by formaldehyde, is not influenced appreciably by the reaction time. With green cabbage essentially all of the reducing capacity of the extract is fixed by formaldehyde. The red cab-

TABLE 3

Results Obtained by Analyzing Beet Roots and Tops for Ascorbic Acid Content Using Formaldehyde Fixation to Permit a Measure of the Interference

	Total apparent ascorbic acid, mg./100 gm. fresh weight	"True" ascorbic acid, mg./100 gm. fresh weight	Per cent of total apparent ascorbic acid not fixed by CH_2O
Beet roots	7.06±0.52[1]	3.18±0.32	54.7±6.8
Beet tops	9.96±0.24	5.97±0.51	40.2±2.97

[1] Mean ± standard error.

TABLE 4

Total Apparent Ascorbic Acid and "True" Ascorbic Acid Contents of Various Plant Material

Product	"True" ascorbic acid, mg./100 gm. fresh weight		Per cent of total apparent ascorbic acid not fixed by CH_2O		Recovery of added ascorbic acid		Average ratio between aliquot sizes[3]
	10 sec.[1]	Av.[2]	10 sec.[1]	Av.[2]	10 sec.[1] *pct.*	Av.[2] *pct.*	
Green cabbage	39.9	40.3	(−0.5)	0.4	96.6	95.4	1.03
Red cabbage	61.9	62.2	4.3	3.7	96.4	94.3	1.06
Lucellus chard	11.4	11.5	3.0	3.8	93.5	92.5	1.08
Ruby King chard	9.7	9.8	8.1	9.8	95.7	91.8	1.10
Elderberries	58.9	59.7	5.1	4.9	93.7	97.0	1.05
Red raspberries	12.1	11.8	10.7	13.6	94.3	92.4	1.06
Blackberries	10.4	10.3	12.9	15.2	100.0	94.6	1.08
Black currants	12.7	11.7	32.7	39.6	88.8	82.2	1.14
Blueberries, wild	8.4	8.0	25.1	30.9	94.1	89.2	1.14
Chokecherries	0.46	0.31	38.9	54.4	86.4	84.0	1.14
Black raspberries	6.6	6.4	31.4	39.9	91.1	84.7	1.21
Blueberries, cult.	3.3	3.0	40.1	44.7	89.8	87.4	1.26

[1] Reaction time.
[2] Average value for 10-, 20-, and 40-second reaction times.
[3] Average ratio between the total apparent ascorbic acid content determined using 2- and 5-ml. aliquots.

bage showed a slightly greater amount of interfering material. A similar comparison can be made between green and red chard. Among the fruits analyzed, elderberries are conspicuous for their relatively low amount of interfering material and high ascorbic acid content. In all of the other fruits the amount of apparent ascorbic acid not fixed by formaldehyde was appreciable. It will be noted in these cases that the recovery of added ascorbic acid tends to be low and that the 2-ml. aliquots show relatively much more total apparent ascorbic acid than do the 5-ml. aliquots. These reaction characteristics are very similar to those obtained with extracts from the beet roots (hypocotyls) and with isolated betanin.

The fact that extracts from elderberries showed so little evidence of reducing materials not fixed by formaldehyde was interesting, since these extracts were highly colored. It appeared possible that the pigments from this fruit might also react with the dye but that the relative amount of ascorbic acid present was so great that the reaction of the anthocyanin was of little consequence in the analysis. This possibility was tested by the preparation of a solution of the anthocyanin (Preparation No. 4, above). The press juice and the final anthocyanin solution contained a reducing capacity toward the indophenol dye equivalent to 27 and 10 mg. of ascorbic acid per ml., respectively. In the press juice, 54 per cent of this reducing capacity remained after formaldehyde fixation; in the final anthocyanin solution the corresponding value was 84 per cent.

From this it appears that the pigment, or pigments, of elderberries will react with the dye, but that in the presence of large amounts of ascorbic acid the magnitude of the interference is small. This indicates that the ratio of interfering material to ascorbic acid is of importance from an

analytical standpoint. This is illustrated further by the data in Table 5. The products described in this table are identical with those listed in Table 4, except that they were stored at —18°C. for various periods of time. The blueberries were stored overnight, the other products for about seven months. They were added directly to the extracting acid without preliminary thawing and the resulting extracts were analyzed in the same manner as that used with the fresh products. Only the averages for the three reaction times are presented. Even green cabbage shows interference to the extent of 15 per cent after being frozen. With the anthocyanin-containing products the magnitude of the interference is much greater.

TABLE 5

Total Apparent Ascorbic Acid and "True" Ascorbic Acid Contents of Some Frozen Anthocyanin-Containing Products

Product	"True" ascorbic acid, mg./100 gm. fresh weight	Per cent of total apparent ascorbic acid not fixed by CH_2O	Recovery of added ascorbic acid
			pct.
Blueberries (cultivated)	0.3	88.3	83.3
Elderberries	4.5	44.6	92.2
Red raspberries	7.3	31.5	92.6
Green cabbage	5.1	15.4	97.0
Red cabbage	3.5	36.3	94.7

On the basis of these results it seems probable that one or more of the pigments present in these anthocyanin-containing plant products react with the indophenol dye used in the determination of ascorbic acid. The reaction is relatively slow. If the time the dye is allowed to react with the extract is carefully controlled and formaldehyde fixation is used, it appears that the magnitude of the anthocyanin reaction can be evaluated. If this is true, a valid measure of the ascorbic acid content of anthocyanin-containing products probably can be obtained. For this purpose the volume of all reactants must be constant, e.g. 5 ml. of tissue extract, 5 ml. of buffer, 5 ml. of the indophenol dye, and, where appropriate, 0.5 ml. of formalin. The reaction time with the dye should be constant; a 20-second reaction time is recommended. Probably the assays in the presence and absence of formaldehyde should be run close together to avoid, insofar as possible, changes in room temperature.

SUMMARY

It has been found that a number of anthocyanin-containing fruits and vegetables contain interfering substances which react with the indophenol dye used in the indophenol-xylene extraction method for determining ascorbic acid. These interfering substances, which appear to be the anthocyanins themselves, react with the dye in the presence of formaldehyde and hence this reaction can be distinguished from that obtained with ascorbic acid. Purified betanin prepared by Pucher, *et al.* (1938) from red garden beets shows reaction characteristics similar to those of the interfering substances from beets and other anthocyanin-containing plant products. Partially purified betanin prepared by an entirely different

procedure shows similar reaction characteristics and does not alleviate scorbutic symptoms in guinea pigs. In the bioassay it was observed that the unsupplemented stock diet could be used instead of the classical Sherman and Smith scorbutogenic diet.

With fresh elderberries the concentration of ascorbic acid is high enough so that the relative interference due to the pigment, or pigments, is negligible. However, by appropriate methods an anthocyanin preparation can be obtained from these berries which reacts with the indophenol dye even in the presence of formaldehyde. In frozen products where the ascorbic acid content is small, relative to the color of the extracts, the interference is serious.

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COLOR AND ASCORBIC ACID VARIATIONS IN CABBAGE COOKED BY VARIOUS METHODS[1]

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The effect of various methods of cooking upon the retention of nutrients in vegetables has long been of interest to the food analyst and dietitian alike. The results reported have been so variable, however, that they have been difficult to interpret. Part of the variability has been caused by changing methods of analysis and differences in the methods of cooking, but much has resulted from a failure on the part of many workers to replicate the cookings. The present paper reports data on the ascorbic acid retention in cabbage during cooking by four commonly used methods and shows the importance of replicate cookings. The effect of the various cooking methods upon the color of the vegetable is reported also.

EXPERIMENTAL PROCEDURE

Varieties and Source of Cabbage. Five varieties of cabbage were used: Marion Market, Danish Ballhead, Golden Acre, Resistant Detroit, and Bugner. They were grown on University plots under supervision of the Horticulture Division. All of the gatherings were analyzed within a few hours after harvest and a few were analyzed also after 24 hours' storage at 5°C.

Preparation and Cooking of Samples. The heads of cabbage from each gathering were matched as nearly as possible according to size and general appearance and were distributed to form three to five similar groups. One of the groups was used for preliminary cookings which were made to find the minimum periods necessary to give a tender product by each of the cooking methods. The other groups were used for replicate cookings.

The details of preparing the cabbage for cooking and analysis were as follows: all of the heads belonging to a single group were cut into five wedge-shaped pieces. One piece from each head was assigned to the raw sample and one to each of the cooking methods. The samples intended for cooking were wrapped in waxed paper and returned to the refrigerator. The wedges in the raw sample were coarsely shredded, the shredded material well mixed, and aliquots removed for ascorbic acid and moisture determinations. The samples reserved for cooking were withdrawn from the refrigerator, one after the other as quickly as they could be handled. In each case the cabbage was coarsely shredded and mixed, then a 215-gm. and a 25-gm. portion were weighed, the latter tied into a loose cheesecloth bag, and both portions cooked together by one of the methods listed below for the length of time decided upon during preliminary cooking tests (Table 1). The vegetable was drained, the contents of the bag emptied immediately into a Waring Blendor containing cold, 3 per cent metaphosphoric acid, and a portion of the cooking water set in an ice bath to cool quickly. The procedure of bagging the ascorbic acid samples places that determination directly on the raw weight basis and eliminates the cooling period (and the possibility of oxidation during that time) which is necessary if small cooked portions are to be weighed with even a moderate degree of accuracy.

The other group of heads were removed from the refrigerator one after the other as rapidly as possible and were prepared for cooking and analysis in the manner just

[1] Scientific Journal Series paper No. 2579, Minnesota Agricultural Experiment Station.

TABLE 1

Length of Cooking Periods

Variety	Crop year	Lot number	Cooking period			
			Open kettle	Tightly covered kettle	Pressure saucepan	Steamer
			min.	*min.*	*min.*	*min.*
Marion Market	1944	1, 2	9.5	25	3.5	37
Marion Market	1945	1, 2	9.5	25	2.0	37
Danish Ballhead	1944	1, 2	12.5	40	8.5	50
Golden Acre	1944	1	5.5	16	2.25	30
Golden Acre	1944	2	9.5	25	3.5	37
Detroit Resistant	1944	1	9.5	25	2.0	37
Bugner	1945	1	9.5	25	2.0	37

described. In most cases the period they remained in the refrigerator amounted to only a few hours but in five cases it amounted to approximately 24 hours (see Table 2).

The cooking methods were as follows: *a*, cooking in four times the weight (i.e. 960 ml.) of boiling tap water; *b*, cooking in a tightly covered pan in just enough water to prevent scorching (60 ml. for all of the varieties except Danish Ballhead, for which the amount was 90 ml.); *c*, cooking on a rack above the water level in a pressure saucepan at 15 pounds' pressure, and *d*, cooking in a steamer.

Four replicate series of samples were prepared, cooked, and analyzed independently of one another.

Methods of Analysis. The ascorbic acid determinations were made on the raw and cooked samples and upon aliquots of the cooking waters by the Morell (1941) modification of the Bessey (1938) method, except that the correction of the latter method for color and turbidity was retained. Moisture was determined by the differences in weight of 10-gm. samples before and after drying for 18 hours in a vacuum oven at 95°C. and 25 inches of mercury. Color was determined by the Nickerson (1929) whirling disc method.

The least significant difference between the means for the percentage retention of ascorbic acid in the cooked samples was calculated. When this criterion indicated no significant difference, the means were further analyzed by the method of Tukey (1949).

RESULTS AND DISCUSSION

The ascorbic acid content of the raw cabbage is given in Table 2. The The values, which lie between 30 and 65 mg. of ascorbic acid per 100 gm. of vegetable, are similar to those given by other workers (Eheart and Sholes, 1948; Hewston, Dawson, Alexander, and Orent-Keiles, 1948; Kelley, Jackson, Sheehan, and Ohlson, 1947; Sutherland, Halliday, and Hinman, 1947; Mayfield and Richardson, 1940; Noble and Waddell, 1945; Quinn, Scoular, and Johnson, 1946; and Van Duyne, Chase, and Simpson, 1944) but are markedly lower than ones reported for a number of varieties grown on silty clay loam fertilized with NKP (Burrell, Brown, and Ebright, 1940).

The percentages of ascorbic acid retained in the cooked samples of cabbage and dissolved in the cooking waters at the end of the cooking periods are given in Table 3. These data show the inadvisability of formulating general statements concerning the effectiveness of different methods of cooking in retaining ascorbic acid when results from only one series of cookings are available. Obviously, the method selected and the relative order of each of the other methods, with the exception of the open kettle method, would depend upon the particular series which happened to have

TABLE 2

Ascorbic Acid and Moisture Content of Raw Cabbage

Variety	Crop year	Lot number[1]	Period between harvest and analysis[2]	Ascorbic acid content	Moisture content
			hours	*mg./ 100 gm.*	*pct.*
Marion Market	1944	1a	5	30.6	93.3
		1b	24	38.0	93.0
		2a	4	37.9	92.3
		2b	24	39.1	92.6
	1945	1a, 1b	1-2	52.1	92.1
		1c, 1d	3-4	52.3	92.2
Danish Ballhead	1944	1a, 1b	2-4	43.2	92.0
		2a, 2b	1-2	40.3	92.3
Golden Acre	1944	1a	2	30.3	93.6
		1b	24	30.9	93.8
		2a	1	34.5	94.2
		2b	5	33.8	93.9
Resistant Detroit	1945	1a, 1b	1-2	45.6	93.4
		1c, 1d	4-5	48.5	92.9
Bugner	1945	1a, 1b	1-2	65.5	91.4
		1c, 1d	22-23	51.2	91.2

[1] The letters following the lot number indicate replicate samples taken from the same gathering.
[2] The heads were stored at 5°C. during this period.

been analyzed. For example, if one were depending only upon the results from lot 1a of Danish Ballhead cabbage, it would appear that cooking in the pressure saucepan was superior to all the other methods tried, while if one had only the figures for lot 2a of the Danish Ballhead cabbage, the conclusion probably would be that cooking in the steamer was the superior method. Actually, as will be shown presently, neither of these methods was superior on the average to the other or to cooking in the covered pan in the retention of ascorbic acid in the Danish Ballhead cabbage.

On the basis of the mean values reported in Table 3 and the least significant differences between them, the vegetable samples cooked in the tightly covered kettle, the pressure saucepan, and the steamer, showed no significant differences in the percentages of ascorbic acid retained, except in two instances. In the case of the 1945 samples of Marion Market cabbage, those cooked in the tightly covered pan averaged slightly, but significantly, lower than those cooked in the pressure saucepan and steamer. Also, in the case of the Bugner cabbage, the samples cooked in the tightly covered kettle and in the steamer averaged slightly, but significantly, lower than those cooked in the pressure saucepan.

Additional analysis of these data by the Tukey method (1949) failed to give any further separation of the means.

All of the samples cooked in the tightly covered kettle, pressure saucepan, and steamer showed considerably higher retentions, however, than did the ones cooked in boiling water. Their retentions, on the average, were between one-half and three-fourths of the amount of the ascorbic acid

TABLE 3

Retention of Ascorbic Acid in Cabbage During Cooking by Different Methods

Variety	Year	Lot number	Ascorbic acid retained in vegetable				Ascorbic acid dissolved in cooking water			
			Open kettle	Tightly covered kettle	Pressure saucepan	Steamer	Open kettle	Tightly covered kettle	Pressure saucepan	Steamer
			pct.	*pct.*	*pct.*	*pct.*	*pct.*	*pct.*	*pct.*	*pct.*
Marion Market	1944	1a	32	95	76	87	46	1	0	6
		1b	29	66	78	68	42	1	2	0
		2a	30	65	71	64	50	5	5	1
		2b	32	78	75	65	45	0	5	0
	1945	1a	34	61	70	70	52	8	10	9
		1b	34	54	66	62	47	10	9	8
		1c	33	63	74	73	53	10	10	9
		1d	36	64	70	65	51	8	11	8
	1944	mean[1]	31	76	75	71	46	2	3	2
	1945	mean[1]	34	61	70	68	51	9	10	8
Danish Ballhead	1944	1a	34	51	74	58	53	1	10	1
		1b	36	50	58	58	45	8	8	4
		2a	35	52	45	64	43	10	6	2
		2b	31	48	51	59	44	6	7	3
	1944	mean[1]	34	50	57	60	46	6	8	2
Golden Acre	1944	1a	36	88	82	88	39	1	1	0
		1b	42	72	69	67	38	0	2	0
		2a	26	60	70	62	35	0	3	0
		2b	29	76	73	73	37	1	3	0
	1944	mean[1]	33	74	74	72	37	1	2	0
Resistant Detroit	1945	1a	40	65	72	73	48	14	11	11
		1b	42	60	74	72	51	10	12	10
		1c	36	58	68	60	46	5	12	6
		1d	28	70	69	70	43	7	11	8
	1945	mean[1]	37	63	71	69	47	9	12	9
Bugner	1945	1a	37	59	71	66	52	4	8	4
		1b	36	59	68	61	40	2	4	4
		1c	31	62	69	63	52	3	7	4
		1d	31	53	79	56	57	7	8	4
	1945	mean[1]	34	58	72	62	50	4	7	4

[1] Least significant difference at 5 per cent level between means for vegetable samples: Marion Market, 1944 crop, 11, 1945 crop, 4; Danish Ballhead, 11; Golden Acre, 11; Resistant Detroit, 8; Bugner, 7.

present in the raw sample while the retention in the boiled samples averaged about one-third of the original amount.

The condensation water from the steamer and the cooking waters from the tightly covered pan and the pressure saucepan contained only small quantities of ascorbic acid, usually considerably less than 10 per cent of the amount present in the raw sample. Those from the open kettle, however, contained a large proportion, usually almost 50 per cent, of the original amount.

The effect of the different cooking methods upon the amount of ascorbic acid present in the cooked vegetable and cooking water together is of interest even though it is usually of little practical value because the cooking waters are ordinarily not used at once, if at all. Statistical analysis

of the results showed that the drained vegetable samples plus the cooking waters from the open kettle and pressure saucepan contained slightly, but significantly, more ascorbic acid than did those from the steamer and tightly covered pan. Thus, the open kettle and pressure saucepan samples contained about 80 per cent of the ascorbic acid originally present in the raw samples, while the steamed and tightly covered samples contained about 70 per cent. It is evident from these figures, however, that a large proportion of the ascorbic acid present in raw cabbage remains unchanged during cooking, regardless of the method used.

TABLE 4

The Hue, Brilliance, and Chroma of Cabbage Before and After Cooking

Variety and method of cooking	Percentage area of color discs exposed				Color notation[1]		
	G 5/6	Y 7/8	N 9.4/	N 1/	Hue[2]	Brilliance	Chroma
Marion Market							
Raw	54	34	8	4	34.2 or 4.2 GY	6.1	6.0
Open kettle	53	34	6	7	34.1 or 4.1 GY	5.9	5.9
Tightly covered pan	22	37	8	33	29.9 or 9.9 Y	5.5	4.3
Pressure saucepan	35	35	12	18	32.0 or 2.0 GY	6.1	4.9
Steamer	13	35	16	36	28.3 or 8.3 Y	5.9	3.6
Danish Ballhead							
Raw	60	38	2	0	34.1 or 4.1 GY	5.9	6.6
Open kettle	70	28	1	1	36.4 or 6.4 GY	5.9	6.4
Tightly covered pan	17	41	5	37	27.6 or 7.6 Y	5.4	4.3
Pressure saucepan	15	36	10	39	28.7 or 8.7 Y	5.7	3.8
Steamer	16	39	8	37	28.6 or 8.6 Y	5.5	4.1
Golden Acre							
Raw	48	40	10	2	32.8 or 2.8 GY	6.4	6.1
Open kettle	53	36	3	8	33.8 or 3.8 GY	5.8	6.1
Tightly covered pan	21	42	8	29	29.3 or 9.3 Y	5.8	4.6
Pressure saucepan	38	39	7	16	31.9 or 1.9 GY	5.9	5.4
Steamer	14	41	5	40	28.1 or 8.1 Y	5.3	4.1

[1] Formulas used for calculation are given by Nickerson (1929).
[2] The higher the figure preceding the letters GY, the nearer the color approaches green.

The color data are given in Table 4. As may be noted from the column giving hue, the open kettle samples of all three varieties were either as green or somewhat more green than the corresponding raw sample. (With the form of color notation employed in Table 4, the higher the figure preceding the letters GY, the nearer the color approaches green.) The pressure saucepan samples of the Marion Market and Golden Acre varieties were considerably less green than the raw, while those of the Danish Ballhead variety and all of the tightly covered and steamed samples lost their greenness completely and retained only their yellow hue. Brilliance was not affected in any consistent way by the method of cooking, while the intensity of the color (chroma) was considerably greater for the open kettle and raw samples than for any of the others.

SUMMARY

1. Changes in color and ascorbic acid content caused by different methods of cooking have been determined with several varieties of cabbage.

2. Samples which were cooked in boiling water were as green or greener than corresponding raw ones, while others cooked in a tightly covered pan or steamer no longer showed any trace of green. Samples cooked in a pressure saucepan were never as green as the raw, but some of them retained some of their original green color.

3. The retention of ascorbic acid varied sufficiently between applications to make it inadvisable to rate methods of cooking upon a single series of cookings.

4. The average amounts of ascorbic acid retained in the samples cooked in a tightly covered pan, a steamer, and a pressure saucepan usually were not significantly different from one another and lay between one-half and three-fourths of the quantity present in the raw vegetable. The retention in the samples cooked in boiling water was approximately one-fourth of the amount in the raw cabbage.

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ASCORBIC ACID IN PEACHES FROM SOUTHERN NEW MEXICO

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It is known that the ascorbic acid values of fruits and vegetables vary with variety and environmental conditions. Light is an especially important factor in determining the ascorbic acid content of plants, and the effect of light on the ascorbic acid values of fruits and vegetables has been studied extensively. This subject was reviewed recently by Somers and Beeson (1948). Higher than average ascorbic acid values have been reported for fruits and vegetables grown in the sunny mountainous areas of the southwestern part of the United States by Smith, Burlinson, and Griffiths (1943), Pyke (1944), and others.

For this reason it seemed worthwhile to determine the ascorbic acid values, during two seasons, of 35 varieties of peaches from the experimental orchards of the New Mexico College of Agriculture and Mechanic Arts. The College is located in a narrow irrigated valley of southern New Mexico in a semi-arid region of abundant sunshine. The altitude is 3800 feet.

The peaches were available during a period of about two and a half months. The earliest varieties were ready for picking the last week in June and the latest about the first week in September. Samples of 5 to 10 lb. of the tree-ripened peaches were obtained at the orchard the day the peaches were picked and taken to the laboratory for analysis.

The peaches were peeled by hand with a stainless steel knife. A composite sample was made of each variety from two slices removed from opposite sides of each peach in the lot. Ascorbic acid was determined in 0.5 per cent oxalic acid or 1 per cent metaphosphoric acid extracts of the peaches by the photoelectric indophenol method described by Bessey (1938) except that the acid extracts were unbuffered as suggested by Ponting (1943). There was no difference in ascorbic acid values in metaphosphoric and oxalic acid extracts of the same peaches.

The ascorbic acid values of the peaches are shown in Table 1. They ranged from 4 to 22 mg. of ascorbic acid per 100 gm. of peeled fruit. Values were much the same in the two years, averaging 11.9 mg. per 100 gm. for 30 samples from 29 varieties in 1946 and 12.3 mg. for 47 samples from 35 varieties in 1947. In general, those varieties which ripened either early or late in the season had higher ascorbic acid values than those ripening in July and early August. The ascorbic acid content of the New Mexico peaches was considerably higher than that reported for some of the same varieties grown in South Carolina by Mitchell, Van Blaricom, and Roderick (1948). Fourteen varieties of South Carolina peaches had an average ascorbic acid value of 5.75 mg. without peeling and 5.00 mg. when peeled. This difference in vitamin C value may be due to the amount of light in the two regions.

TABLE 1

Ascorbic Acid Content of New Mexico Peaches

Variety	Color of flesh	1946 crop		1947 crop	
		Date	Ascorbic acid	Date	Ascorbic acid
			mg./ 100 gm.		*mg./ 100 gm.*
Early Wheeler	White	June 24	22	June 30	19
				July 1	17
Early Red-fre	White	June 24	20	June 30	12
				July 1	14
June Elberta	Yellow	June 24	18	June 30	12
				July 1	13
Fisher	Yellow	July 1	17	June 30	10
				July 1	8
Golden Jubilee	Yellow	July 8	8	July 9	9
		July 15	10	July 14	9
				July 15	6
				July 16	4[1]
				July 21	9
Red Haven	Yellow			July 15	14
Vedette	Yellow	July 15	14	July 21	11
				July 26	11
Alton	White			July 21	12
				July 23	13
President Roosevelt	Yellow	July 18	13	July 21	14
Frankie	Yellow	July 18	16	July 24	13
Golden East	Yellow			July 24	12
Fire Glow	Yellow	July 22	8	July 24	8
July Elberta	Yellow	July 15	13	July 25	15
Golden Globe	Yellow			July 25	13
Valiant	Yellow	July 24	15	July 26	12
				July 29	9
South Haven	Yellow	July 22	12	July 28	11
July Gold	Yellow	July 22	16	July 29	15
Colora	Yellow	July 22	10	July 29	12
Halehaven	Yellow	July 22	14	July 29	10
Burbank Giant	Yellow	July 23	13	Aug. 1	15
Hardee	Yellow	July 31	8	Aug. 4	9
Early Elberta	Yellow	Aug. 9	9	Aug. 4	10
Hiley	White	July 31	13	Aug. 6	18
Elberta	Yellow	July 29	9	Aug. 6	14
Golden Elberta Cling	Yellow	July 29	8	Aug. 6	9
				Aug. 8	8
J. H. Hale	Yellow	Aug. 10	7	Aug. 11	11
Hardy Berta	Yellow	Aug. 10	12	Aug. 11	11
Halberta	Yellow	Aug. 10	10	Aug. 11	10
Giant Snowball	White	Aug. 9	16	Aug. 13	18
Rio Oso Gem	Yellow	Aug. 9	6	Aug. 14	12
Late Elberta	Yellow			Sept. 3	15
Salberta	Yellow	Aug. 19	6	Sept. 4	19
Frank	Yellow	Aug. 19	5	Sept. 4	16
Halate	Yellow	Aug. 19	10	Sept. 5	17
Dr. Burton	Yellow			Sept. 6	18
Mean: 30 samples				47 samples	
29 varieties			11.9	35 varieties	12.3

[1] Very ripe.

SUMMARY

Ascorbic acid was determined during two seasons in 35 varieties of peaches grown in southern New Mexico. Ascorbic acid values ranged from 4 to 22 mg. per 100 gm. of peeled fruit. These values are higher than are usually reported for this food. It is suggested that the high values may be due to the abundant sunshine of the region.

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EFFECT OF TWO HEAT TREATMENTS ON RANCIDITY DEVELOPMENT IN UNSHELLED PECANS[1]

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The kernels of newly harvested pecans are crisp, fresh, and appetizing. The kernels of those available during the summer and early fall are often dry and strong in flavor. Sometimes the flavor is markedly rancid. It is generally agreed that autoxidation of a fat is the major cause of rancidity. Many factors are known to accelerate autoxidation. Coe (1941) classifies them as the primary factors such as metals and light, and the contributory factors such as air, moisture, temperature, photosensitizers, enzymes, etc. Other workers have shown that rancidity can be retarded in pecans by cold storage, by removal of oxygen from the storage atmosphere, and by partial desiccation of shelled kernels (Baker, 1938; Blackmon, 1933; Brison, 1945; Harris, 1942; Medlock, 1933; Wright, 1932, 1941). In an effort to find a simple method of retarding rancidity in unshelled pecans, two heat treatments were developed in this investigation. The effects of the heat treatments on enzymatic activity and rancidity retardation were observed.

EXPERIMENTAL PROCEDURE

Pre-storage Treatments of Pecans. Experiments were conducted to determine a procedure for heating nuts which would inhibit enzyme activity, yet still retain desirable eating characteristics. Since most enzymes are destroyed at 80°C.(176°F.) (Sumner and Somers, 1943) the pecans were heated to attain this internal temperature. This temperature was determined by the use of sample nuts, one of which was placed in the center of the pan of pecans being heated. A hole was bored through the shell of each sample pecan and a thermocouple inserted into the thickest portion of the kernel.

It was thought that by limiting the air available to the cells, thus restricting the exchange of residual and atmospheric oxygen, rancidity might be further retarded. Hence a method of sealing the pores of the heat-treated pecans by submersion in hot mineral oil was developed. Throughout the study this is referred to as hot oil treatment and the one described above as hot air treatment.

Some unshelled Stuart pecans held in ordinary storage until March first were treated and some were stored without treatment. Two lots were subjected to heat treatments; one lot received no treatment and served as a control. All lots were stored in tightly covered paraffin-lined tin buckets in an unheated room. A total of eighteen sample lots were stored in order that six replications of tests on pecans from the three lots could be made. The two heat treatments used were the hot air and the hot oil treatments developed in this study.

In the hot air treatment the pecans were heated in an ordinary oven at 177°C. (350°F.) until the internal temperature of a sample nut reached 80°C.(176°F.). This heating process required 10 to 12 minutes. The nuts were rapidly cooled in front of an oscillating fan and were stored when cooled to internal temperature of approximately

[1] This paper is an abstract of the thesis submitted by Josephine B. McGlamery in partial fulfillment of the degree of Master of Science in Home Economics at the University of Georgia, August 1949.

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28°C.(82°F.). In the hot oil treatment, the pecans were heated in an oven to the internal temperature of 80°C., then were immediately submerged in a bath of mineral oil at 80°C. for 60 seconds. The nuts were drained and cooled to approximately 28°C. before storing.

Tests Made on Pecans and Pecan Oil. The pecans were tested initially and after 8 weeks', 16 weeks', and 24 weeks' storage. The samples were randomized for testing. Kernels were removed from the shells and the percentage kernel of the nut was calculated. The moisture content of the kernels was determined by the official methods of the Association of Official Agricultural Chemists. Qualitative peroxidase tests were made on the kernels with 1 per cent guiacol solution and 3 per cent hydrogen peroxide solution.

A panel of judges used weighted adjectives in evaluating the flavor and moistness of the pecans. Samples for palatability were made up of chopped kernels from four or more pecans.

For oil extraction each sample of nuts was ground to a homogeneous mixture in a Waring blendor. A 20-gm. sample of the homogenate was placed in a Soxhlet and extracted with 100 ml. of chloroform for three hours. The extract was then brought to 100 ml. volume with chloroform in a volumetric flask. Aliquots were dried to constant weight, and the extracts were refrigerated (8°C., 46°F.). The percentage yield of oil was thus computed. Aliquots of the extracts were used for modified Kreis tests (Watts and Major, 1946) and peroxide numbers (Wheeler, 1932). The Kreis test was made within 2 hours after the oil was extracted and the peroxide tests were made within 24 hours after extraction.

Data from the subjective tests and from the chemical tests were each analyzed at each of the storage periods by analysis of variance. Wherever significant differences were found, further analyses (t-tests) were made to reveal the differences in pre-storage treatments at each time interval.

RESULTS AND DISCUSSION

The progression of rancidity development was more rapid in the untreated lot of pecans than in either of the two treated lots of pecans. Very little difference was apparent between the two heat treatments.

The pecans which had received the heat treatments showed significantly less decline in palatability at the advanced stages of storage than the untreated nuts showed (Figure 1). At the end of the 16- and 24-week storage periods, the hot oil-treated nuts were scored significantly higher than the hot air-treated nuts.

Since flavor is the principal palatability factor in pecans affected by rancidity, analysis was made of the flavor scores of the pecans. At every storage interval the untreated pecans were scored significantly lower than the treated pecans. After 24 weeks of storage, no rancid flavor had developed in either of the two treated lots of pecans, but the flavor of the untreated nuts was markedly rancid (Table 1).

TABLE 1

Total Palatability and Flavor Scores of Stored Pecans

Lots	Total palatability, means				Flavor, means			
	Weeks' storage				Weeks' storage			
	0	8	16	24	0	8	16	24
Untreated	25.0	17.7	12.7	10.4	17.0	11.8[1]	8.4[1]	5.5[1]
Hot air treatment	21.5	19.1	16.1	16.1	16.5	13.9	11.0[1]	11.0[1]
Hot oil treatment	23.0	20.1	20.9	18.4	17.0	13.4	14.2	12.3

[1] Score evaluated 12 was described as stale; 8 was described as slightly rancid.

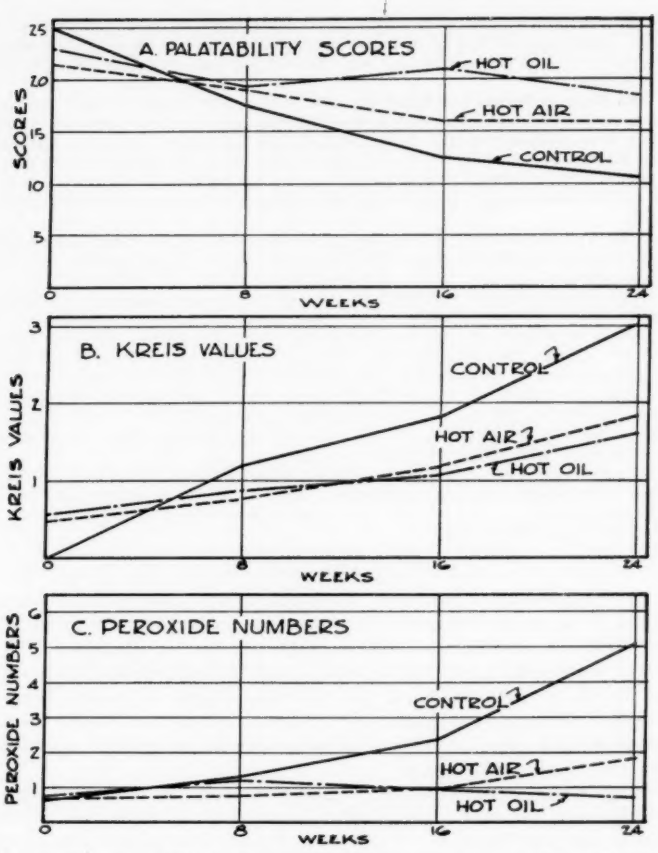

FIG. 1. Rate of rancidity development in Stuart pecans as measured by (A) palatability scores, (B) Kreis values, and (C) peroxide numbers.

Although it was known that heat would tend to make the kernel of the pecan dry, it was believed that if moisture loss was the only deleterious effect of the heat, the nuts could be made acceptable. Wright (1941) has found that pecans may usually be restored to the desired texture by adjusting the storage atmosphere to the proper humidity. A tasting panel of judges found no evidence of a cooked flavor in the kernels subjected to the heat treatment.

The method of extraction of the oil from the kernels yielded an average of 65 per cent oil which indicates that a high percentage, if not all, of the oil was extracted.

Kreis values for the oils of each lot of pecans increased as the length of storage increased. Initial Kreis values for the untreated lots of nuts were too low to be read on the photoelectric colorimeter, but low values were also obtained from the two lots of treated nuts (Table 2). The heat treatment probably accounts for this difference. At the 16-week storage interval, the oil from the kernels of the untreated pecans had significantly higher Kreis values than the oils from either of the two treated lots. Although the oil from the hot air-treated nuts had consistently higher values than the oil from the hot oil-treated nuts, there was no significant difference between the two lots. Differences in values increased among lots in the same order until the end of the study.

TABLE 2

Oxidative Changes in Fats Extracted from Stored Pecans

Lots	Kreis values, means				Peroxide numbers, means			
	Weeks' storage				Weeks' storage			
	0	8	16	24	0	8	16	24
Untreated	0.0	1.2	1.8	2.9	0.6	1.4	2.4	5.1
Hot air treatment	0.5	0.8	1.2	1.8	0.7	0.8	1.0	1.8
Hot oil treatment	0.6	0.9	1.1	1.6	0.8	1.3	1.0	0.4

Peroxide numbers of the oils of the untreated pecans and the hot air-treated nuts increased as the length of the storage period increased. The peroxide numbers for the oils of the hot oil-treated pecans increased until a peak was reached after which the values declined (Figure 1). After 16 and 24 weeks of storage the peroxide numbers of the oils of the control were significantly higher than the peroxide numbers of the oils from either of the two lots of treated pecans. Peroxide numbers indicated no significant difference between the oils from the two treated lots of pecans until a decline in peroxide numbers of the oils from the hot oil-treated pecans became apparent after the end of the 16 weeks of storage. It is expected that the limited supply of oxygen available within the nut which had been dipped in the oil accounted for this behavior. According to Lea (1939), whenever a limited supply of oxygen is available the formation of peroxide will gradually fall off as the free oxygen is consumed.

Since significant differences were found in flavor scores before they were found in the chemical tests, it would seem that rancidity development in the pecans was detected by flavor before the chemical products of oxidation of the fat increased to an appreciable extent.

Moisture content of the kernels of the pecans was reduced by the heat treatments from 4.3 per cent to 3.0 per cent. The reduction in moisture content may have contributed to the retardation of rancidity development in the heat-treated pecans. Harris (1942) reported that pecan kernels with a moisture content of 2 per cent kept fresh at room temperature for two years.

Qualitative peroxidase tests indicated that the peroxidase activity had been markedly curtailed in both the hot air- and hot oil-treated pecans.

There seemed to be little difference in the peroxidase activity of the hot air- and the hot oil-treated pecans; hence, it is possible that the differences in rancidity development exhibited between these two lots may be attributed to the limited supply of oxygen available within the oil-dipped nuts.

SUMMARY AND CONCLUSIONS

Either of two heat treatments tested in this study may be applied as late as four months after harvest to retard rancidity development in unshelled Stuart pecans. Nuts heated to an internal temperature of 80°C. (176°F.) and cooled rapidly or immediately dipped in mineral oil (80°C.) before cooling were less rancid than an untreated lot, when rancidity was measured by palatability scores, Kreis values, and peroxide numbers. This difference was evident for flavor scores after 8 weeks of storage; for Kreis and peroxide numbers after 16 weeks of storage. After 24 weeks of storage no rancid flavor had developed in either of the two heat-treated lots of pecans, but the flavor of the untreated nuts was markedly rancid.

There was no difference between the effectiveness of the two heat treatments in delaying rancidity development as measured by chemical tests. Palatability scores were higher for the hot oil-treated pecans than for the hot air-treated pecans after 16 and 24 weeks of storage.

The two heat treatments inhibited enzyme activity as measured by peroxidase tests. The mean moisture content of the kernels was reduced by the heat treatments from 4.3 per cent to 3.0 per cent. Neither of the two heat treatments had a deleterious effect on the palatability of the pecans.

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VITAMIN LOSSES IN DRIP OBTAINED UPON DEFROSTING FROZEN MEAT

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Cook, Love, Vickery, and Young (1926) found that "drip," the blood-like fluid exuding from frozen meat upon thawing, contained approximately 9 per cent protein and that the percentage of protein was approximately the same for drip from either pork, lamb, beef, or veal. Although this would indicate that a considerable amount of the nutrients in the meat may be lost if the drip is discarded, as is frequently the case, very little information is available on the nutritive value of the drip.

A number of investigators (Ramsbottom and Koonz, 1939; Hiner, Madsen, and Hankins, 1945) have reported that rapid freezing greatly reduces drip losses as compared with slow freezing. Using beef aged 6 days, Pearson and Miller (1950) found that the amount of drip did not appear to be greatly influenced by rate of freezing but increased as the period of freezer-storage was prolonged. However, no indication was found in the literature as to the nutritive value of drip. Thus, this study was undertaken to ascertain the percentage of thiamine, riboflavin, niacin, pyridoxine, pantothenic acid, and folic acid that is lost when drip obtained from frozen defrosted beef is discarded.

EXPERIMENTAL PROCEDURE

The meat used in this study was obtained from a purebred Hereford steer approximately 20 months of age. The steer dressed 57.78 per cent and the carcass graded "U. S. Good." After aging the carcass for 5 days at a local packing plant, the wholesale rib from each side of the carcass was removed and cut into steaks approximately 1½ inches in thickness. Each steak was wrapped in cellophane with the "drug-store" wrap and overwrapped with ordinary butcher paper. After wrapping, the steaks were frozen and stored in a home-freezer at 0°F.

Steaks were removed from freezer-storage as needed for analysis. On removal for analysis, the *longissimus dorsi* muscle was dissected from the remainder of the steak and thawed for 14 to 15 hours at approximately 26°C. in a large funnel, which drained into a graduate cylinder. The *longissimus dorsi* muscle was weighed, both before and after thawing, and the drip was measured to the nearest 0.1 ml.

The *longissimus dorsi* muscle was removed from another steak, and a portion of the frozen muscle was weighed and made into a slurry in a Waring Blendor. This sample was analyzed in order to ascertain the amount of each vitamin in the frozen meat.

Both the drip and meat were hydrolyzed by the same method for each vitamin in order to remove the possibility of variation due to differences in the treatment. The level of each vitamin was determined microbiologically in both the meat and drip.

Thiamine was measured by the gravimetric response of *Phycomyces blakleeanus* according to the method of Hamner, Stewart, and Matrone (1943). Riboflavin was measured

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according to the procedure of Snell and Strong (1939) and as modified in the Difco Manual (1948). The method of Krehl, Strong, and Elvehjem (1943), as outlined in the Difco Manual (1948), was used in determining the amount of niacin. Pyridoxine was determined by the method of Barton-Wright (1945) according to the modification in the Difco Manual (1948). Pantothenic acid was measured by a modification of the method of Skeggs and Wright (1944) as outlined in the Difco Manual (1948). Folic acid was determined according to the modification of the method of Capps, Hobbs, and Fox (1948) as explained in the Difco Manual (1948).

The total amount of each vitamin was determined for both the drip and the frozen meat, and the percentage of each vitamin that emerged in the drip was calculated.

RESULTS AND DISCUSSION

Level of Vitamins in the Meat and Drip

Thiamine. The meat contained 1.00 μg. of thiamine per gm. of fresh meat (Table 1), which is within the range of 0.8 to 1.7 μg. per gm. reported for rib roasts by Cover, McLaren, and Pearson (1944). The drip contained 1.13 μg. of thiamine per ml. Since the specific gravity of the drip was 1.004, the results indicate that the drip was equivalent or slightly superior to fresh beef as a source of thiamine. Upon calculating the percentage of thiamine in the drip, it was found that 12.23 per cent of the total amount of thiamine in the meat was lost in the drip.

Riboflavin. The riboflavin content of the meat was 1.59 μg. per gm. (Table 1), which is in agreement with the range of 1.0 to 2.2 μg. per gm. reported by Cover, *et al.* (1944). The riboflavin content of the drip was 0.74 μg. per ml. Thus, the drip contained less than half as much riboflavin as the meat on a basis of weight. However, the percentage lost in the drip amounted to 10.31.

Niacin. The niacin content of the meat was 34.81 μg. per gm. (Table 1), which compares with the range of 28 to 60 μg. per gm. for beef rib roasts reported by Cover, *et al.* (1944). The drip contained 62.57 μg. per ml., which was nearly as potent as the meat. The calculated losses in the drip amounted to 14.51 per cent of the total amount of niacin in the meat.

Pyridoxine. The pyridoxine content of the meat was 3.48 μg. per gm., which is in good agreement with the values reported by Waisman and Elvehjem (1941) for beef round steak. The drip contained 2.16 μg. of pyridoxine per ml. (Table 1). Upon calculating the percentage of pyridoxine lost in the drip, it was found to be 9.41 per cent of the total amount in the meat.

TABLE 1

B-Complex Vitamins in Frozen Meat and Drip, and the Percentage of Vitamins Lost in the Drip[1]

	Thiamine	Riboflavin	Niacin	Pyridoxine	Pantothenic acid	Folic acid
Drip (μg./ml.)	1.13	0.74	62.57	2.16	29.63	0.08
Meat[2] (μg./gm.)	1.00	1.59	34.81	3.48	9.11	0.15
Per cent of vitamin in drip[3]	12.23	10.31	14.51	9.41	33.26	8.07

[1] All values on a wet basis. [2] The values for meat are for the *longissimus dorsi* muscle analyzed in the frozen condition. [3] Calculations were made by the following formula:

$$\text{Per cent of vitamin in drip} = \frac{\text{Volume of drip (ml.)} \times \mu\text{g. of vitamin per ml. of drip}}{\text{Wt. of frozen steak (gm.)} \times \mu\text{g. of vitamin per gm. of meat}} \times 100.$$

Pantothenic Acid. The meat contained 9.11 μg. of pantothenic acid per gm., which is within the range of 2.10 to 10.00 μg. per gm. reported by Waisman and Elvehjem (1941) for beef round. The content of pantothenic acid in the drip amounted to 29.63 μg. per ml., over three times as much as in the meat. The losses in the drip amounted to 33.26 per cent of the total amount of pantothenic acid in the meat (Table 1).

Folic Acid. The folic acid content of the meat was 0.15 μg. per gm., which compares favorably with the values of 0.11 to 0.17 μg. per gm. reported for beef rib by Schweigert, Pollard, and Elvehjem (1946). The drip contained 0.08 μg. of folic acid per ml. (Table 1). The amount of folic acid found in the drip amounted to 8.07 per cent of the total amount in the frozen meat.

SUMMARY

The "drip" obtained upon thawing of frozen beef contained considerable amounts of the B-complex vitamins. The losses amounted to 12.23 per cent of the thiamine, 10.31 per cent of the riboflavin, 14.51 per cent of the niacin, 9.41 per cent of the pyridoxine, 33.26 per cent of the pantothenic acid and 8.07 per cent of the folic acid. These results indicate the importance of collecting and utilizing the drip obtained upon thawing frozen meat.

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